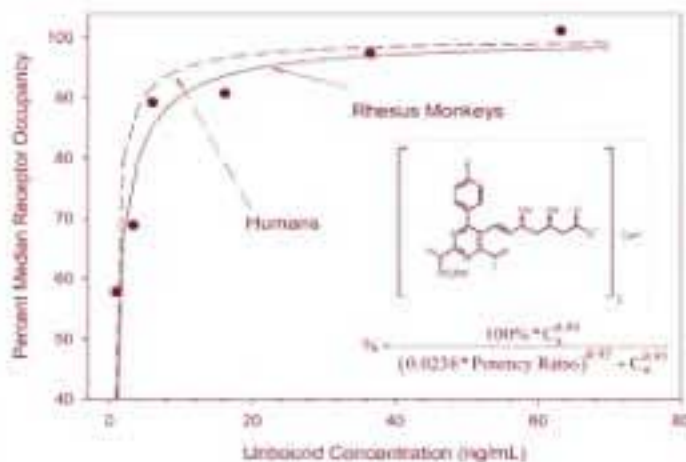


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Foreword

Selection of drug molecules for development from an abundance of potential candidates is a key strategic and risky decision that every company in the business must make routinely. Informative molecule assessment programs will logically facilitate more productive and successful preclinical and clinical development programs, and help guard against unexpected pharmacokinetics or toxicities in clinical testing. Each molecule is unique with its own inherent attributes of efficacy and safety. During the past decade, pharmaceutical scientists have employed standard, as well as breakthrough, technologies in molecular biology and combinatorial chemistry that are customized for a given molecular/therapeutic family in order to significantly increase the rate at which optimal drug candidates progress into preclinical development.

Coincident with the rapid advances in drug molecule discovery, there has been a tremendous expansion in the different scientific domains and skill sets that define preclinical drug development. Experimentally, *in vitro* predictive methods for assessing tissue permeability, cellular transport and organ toxicity have reduced the number of animals used in preclinical drug development while providing more mechanistic insights into the processes of absorption and disposition, and the safety properties of the molecule. Likewise, the technology and underlying science of pharmacogenomics and toxicogenomics has increased the precision of our understanding of drug metabolism and transport processes at the molecular level and the biological responses that up until now were considered to be imprecise because of random variability or noise. Looking to the future, both drug discovery and preclinical development can anticipate an even faster technology evolution as new quantitative and qualitative technologies that assess biological processes and events become more widely available.

Yet, the work is not done. What remains constant is the need for timely, more predictive and cost effective in vitro and in vivo technologies that enable rapid, more accurate, and information-rich decision making to sustain a healthy and successful development pipeline. It is imperative that candidate drug molecules periodically pass through decision gates pre-defined by carefully selected criteria as a strategy to terminate drug development as early as possible where appropriate to reduce unexpected late clinical stage failure. Early and appropriate “no-go” decisions, in particular, provide important opportunities for other more viable candidates to move forward in the pipeline.

This textbook has been specifically written to provide medicinal chemists, biologists, pharmacologists, toxicologists, and academic faculty or students who have an interest in the science of drug development with a better understanding of the preclinical drug development process. By understanding the various components and nuances of preclinical drug development, and the criteria for advancing a candidate molecule into development including clinical trials, the likelihood of success—that is, the commercialization of a truly safe and efficacious drug—will be optimized based on good scientific rationale.

The reader is encouraged to consider this text as a starting point, either as a primer or as a refresher on the basic framework for preclinical drug development. The reader is also encouraged to read similar texts that may be focused selectively on more advanced aspects of the individual topics presented in this book.

Lawrence Lesko, Ph.D.
Director, Office of Clinical Pharmacology
and Biopharmaceutics
Food and Drug Administration
Rockville, Maryland, U.S.A.

Preface

The primary elements of preclinical drug development—product absorption, disposition, and safety—encompass a multitude of diverse disciplines. As such, the preclinical project teams charged with developing a drug candidate are often composed of scientists with expertise in chemistry, biology, pharmacology, pharmacokinetics, and safety. As a team member, you are faced with more questions than answers, technologies that sometimes fall short of demands, the opportunity to improve or even save lives, while always being responsible for the safe and ethical progression of a drug candidate into and through clinical trials. This challenge and the passion for success exist in a team environment where few answers are self evident. As editors, we hope you find this text valuable in generating solutions to the many questions that arise during preclinical drug development.

The scope of this text covers the general elements of preclinical drug development and introduces the reader to these scientific disciplines. Specifically, we introduce you to the framework and rationale for preclinical drug development. You will then find thoughtful discussions on appropriate selection and use of animal models, limits on interpretation of data, and extrapolation of results to potential human outcome. Physiology as it relates to drug absorption, transport, distribution, and elimination is followed by chapters on safety assessment. Liberal use of examples illustrates the application of these principles and technologies. Recognition of the regulatory science and expectations is emphasized.

This text is a starting point for understanding the scientific foundation needed to move a drug candidate into clinical trials and to ensure that the clinical trials proceed efficiently and without unnecessary risk to the patient. You may find additional textbooks that are more focused and advanced on specific scientific disciplines as complementary and we encourage you to learn and develop a practical understanding of drug development. With that

foundation, you will then provide the best value to the decision process; you will optimize the likelihood of developing a safe and effective therapeutic agent.

Completing this project has required significant time, work, and the cooperation of the many chapter authors. We are indebted to those who made these important contributions. We are also indebted to our colleagues and mentors who have served as such great role models. It is not necessary to name you; our admiration and respect for you is evident.

Our employers and supervisors deserve recognition for allowing us to complete this project. Our families deserve special acknowledgment for their strong support and endless patience. Thank you.

Mark C. Rogge, Ph.D.
David R. Taft, Ph.D.

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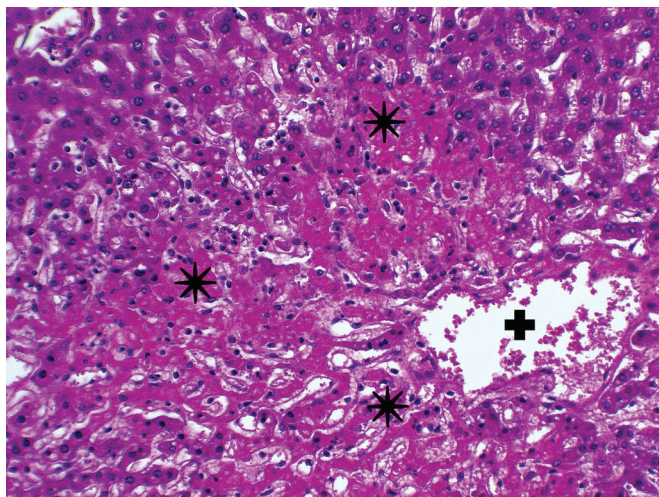


Figure 11.1 Liver: Focal necrosis—A focus of homogenous eosinophilic (pink) necrotic hepatocytes (*) adjacent to a lobular central vein (+). Note loss of normal hepatocellular structure and round nuclei, while elongated nuclei of sinusoidal cells lining the blood spaces are still present.

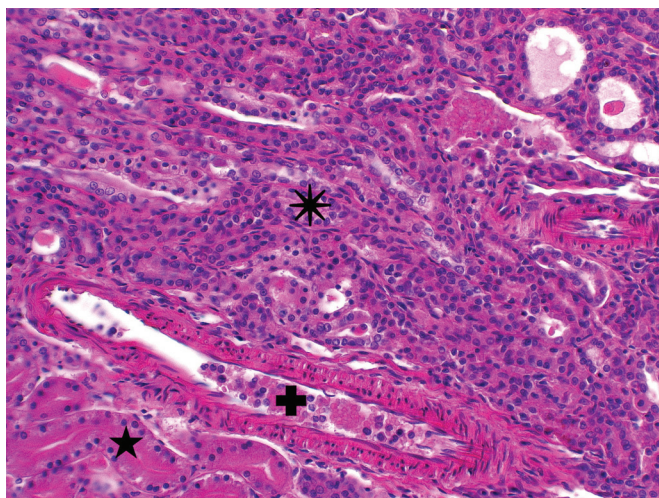


Figure 11.2 Kidney: Regenerating proximal renal tubules—In contrast to normal eosinophilic tubules (★), regenerating tubules (*) are basophilic (blue) and cellular (higher cell density). On this microphotograph normal and regenerating tubules are separated by a renal artery (+).

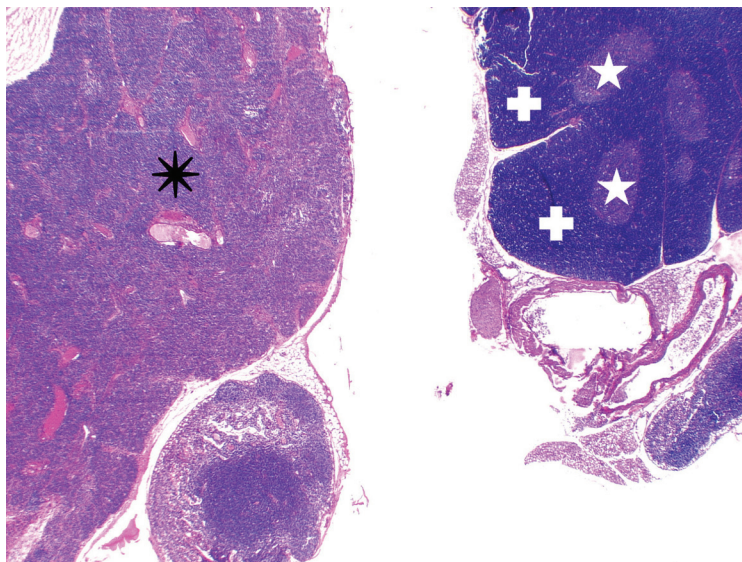


Figure 11.3 Thymus: Atrophy (*) — Note loss of structure in comparison with normal thymus, where cortex (+) and medulla (★) can be clearly distinguished.

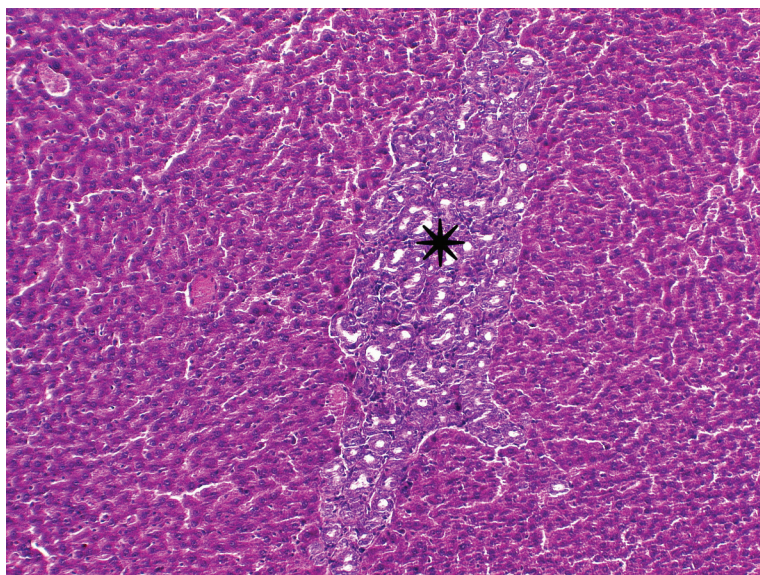


Figure 11.4 Liver: Bile duct hyperplasia—A focus of proliferating bile ducts (*) surrounded by normal hepatic parenchyma.

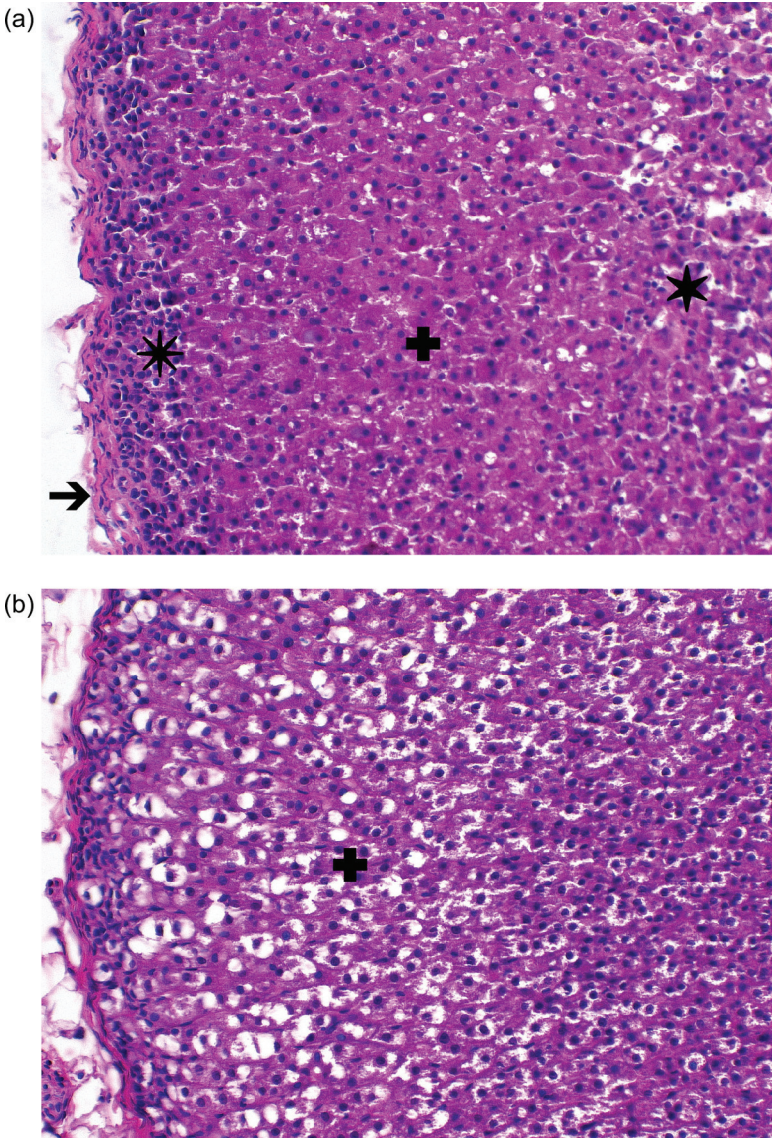


Figure 11.5 (a) Adrenal cortex: Normal—Note the appearance of the three cortical zones, namely the zona glomerulosa (*), zona fasciculata (+), and zona reticularis (*). → = adrenal capsule, and (b) Adrenal cortex: Cortical hypertrophy—Cells of the zona fasciculata (+) are enlarged and vacuolated (optically empty spaces, as the lipid was removed during histological processing).

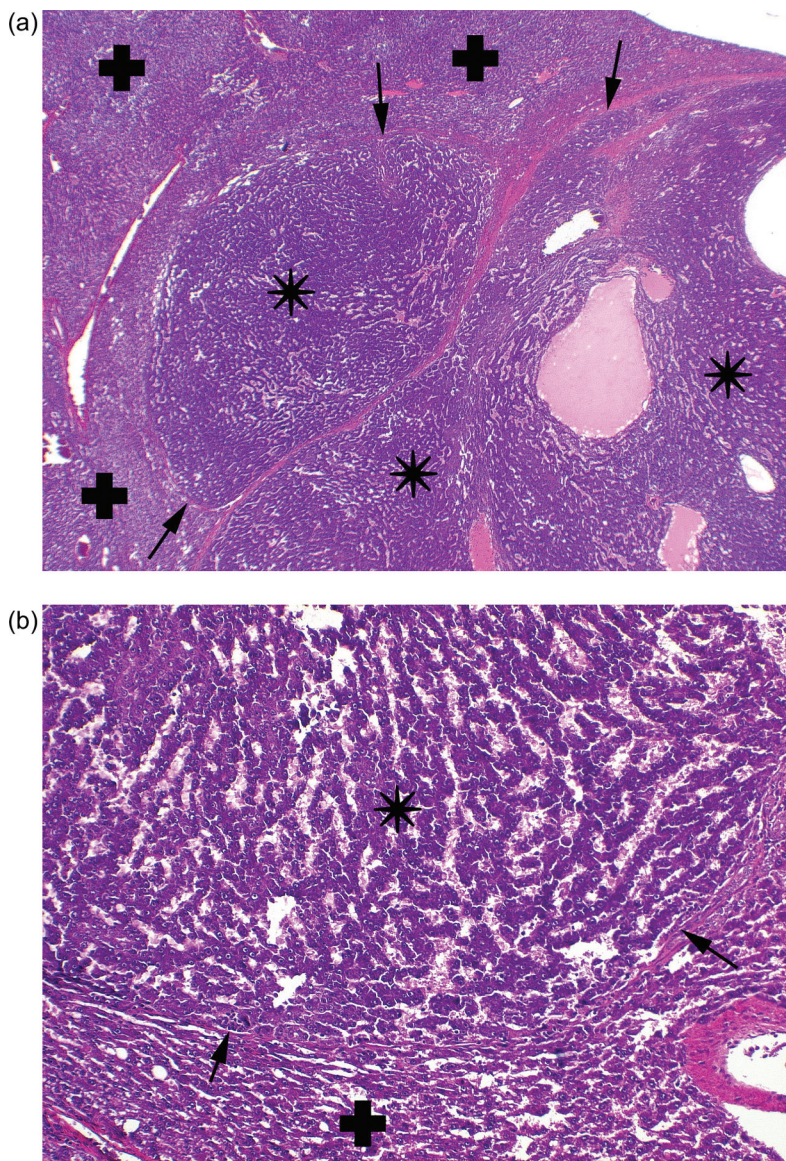


Figure 11.6 (a) Liver: Adenoma (low magnification)—Large areas of proliferating cells (*) with some sinusoidal structure (vascular spaces), compression of normal hepatic parenchyma (+) and partial capsule formation (→), and (b) Liver: Adenoma (higher magnification)—Note the clear delineation between adenoma (*) and normal compressed parenchyma (+). Fibrous strands of early capsule formation (→).

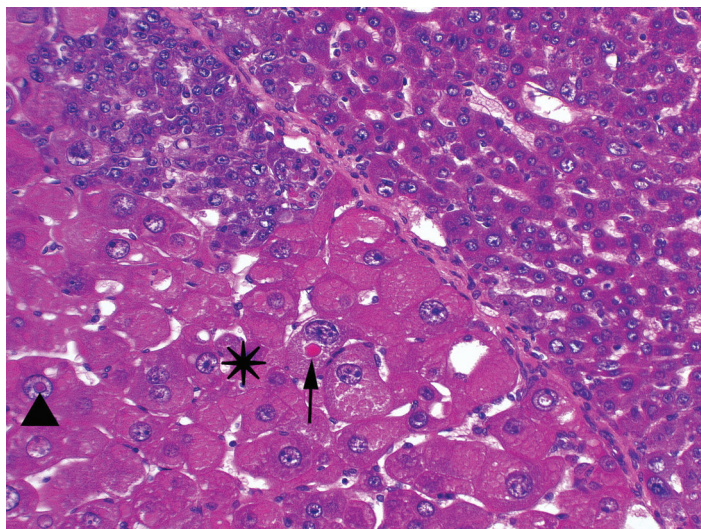


Figure 11.7 Liver: Carcinoma—High magnification of a hepatic carcinoma (*) showing mostly large eosinophilic (pink) tumor cells with nuclei of variable size (nuclear pleomorphism), intranuclear (▶) and intracytoplasmic (→) inclusions and lack of clear sinusoidal structure. Mitotic figures are not apparent on this photomicrograph.

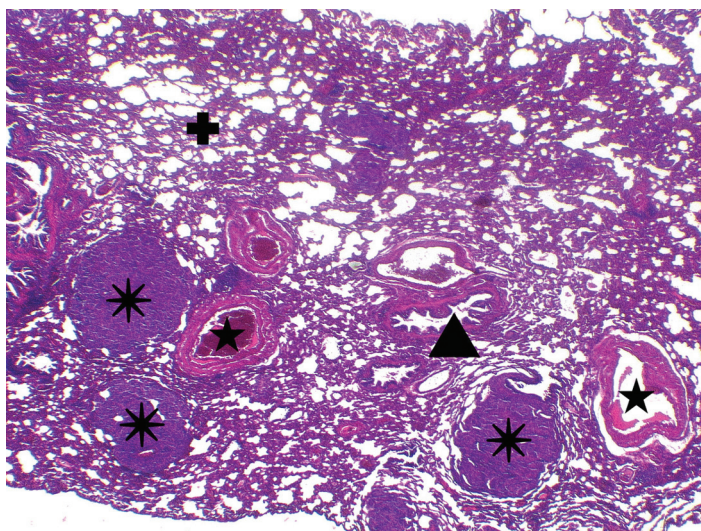


Figure 11.8 Lung: Metastases of fibrosarcoma (*)—Note also normal lung parenchyma (+), large blood vessels (☆), and airways (▶).

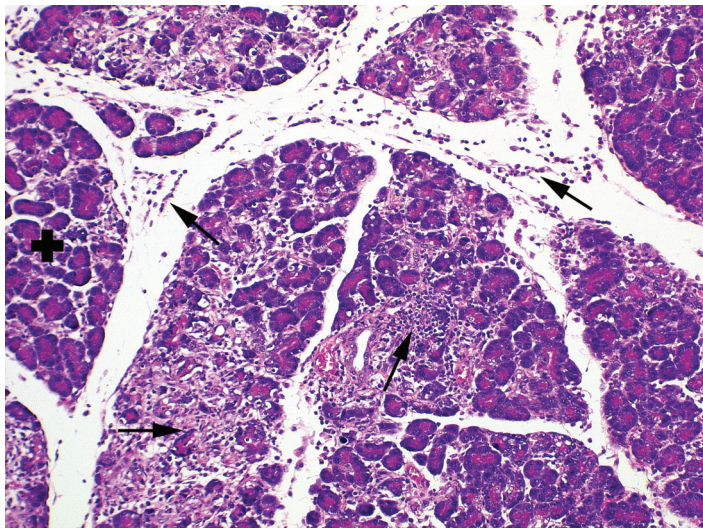


Figure 11.9 Pancreas: Inflammation—Inflammatory cells (→) infiltrating into and around pancreatic lobules. Note also some exocrine atrophy in comparison with normal portion of a lobule (+).

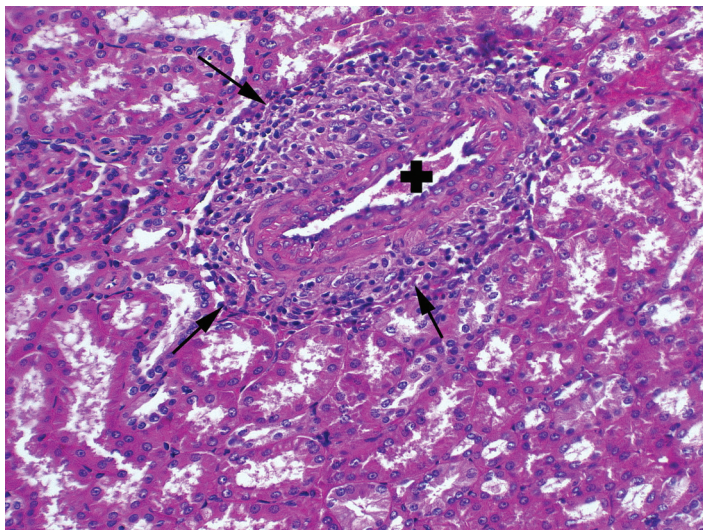


Figure 11.10 Kidney: Vasculitis—Inflammatory cells (→) mainly around a small blood vessel (+). The surrounding renal tubules are normal.

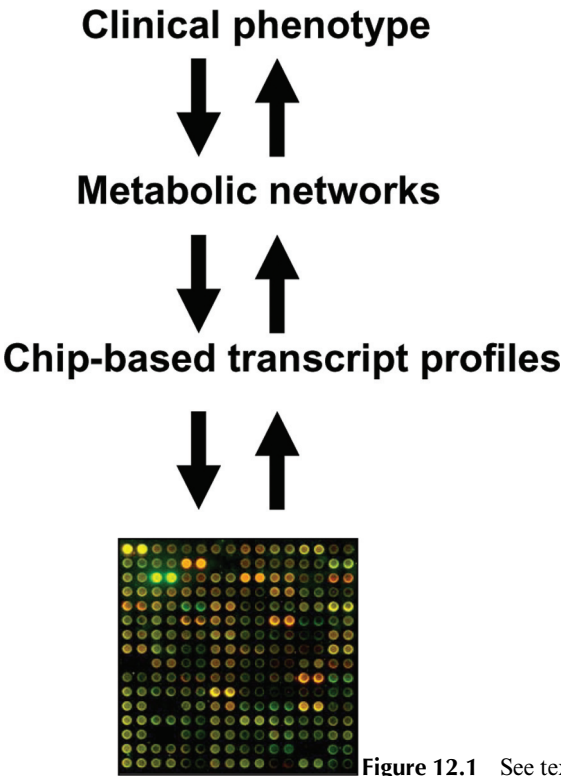
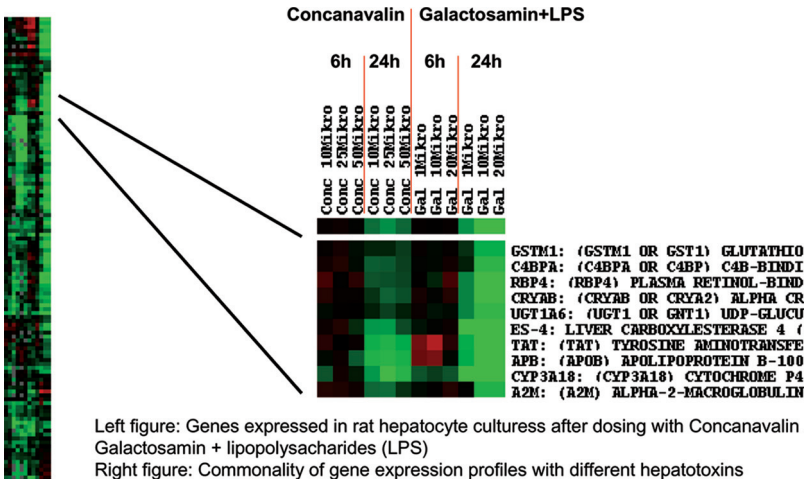


Figure 12.1 See text [page 533](#) for full discussion.



Left figure: Genes expressed in rat hepatocyte cultures after dosing with Concanavalin A or Galactosamin + lipopolysaccharides (LPS)
Right figure: Commonality of gene expression profiles with different hepatotoxins

Figure 12.3 See text [page 535](#) for full discussion.

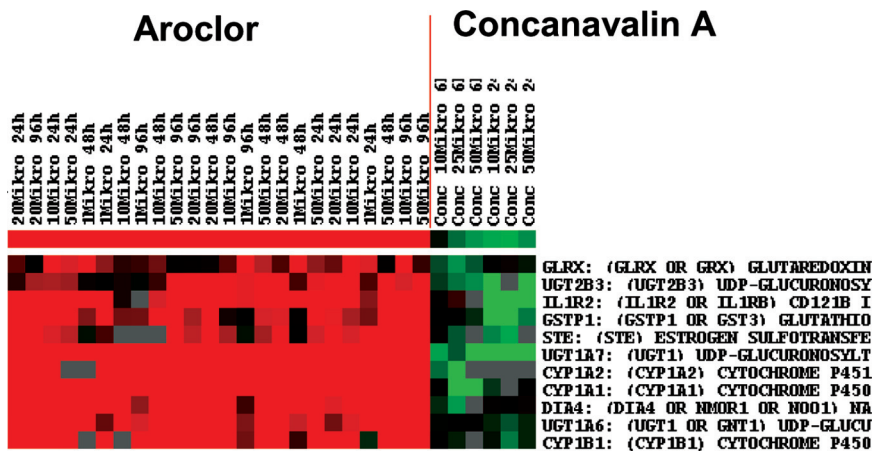


Figure 12.4 See text [page 536](#) for full discussion.

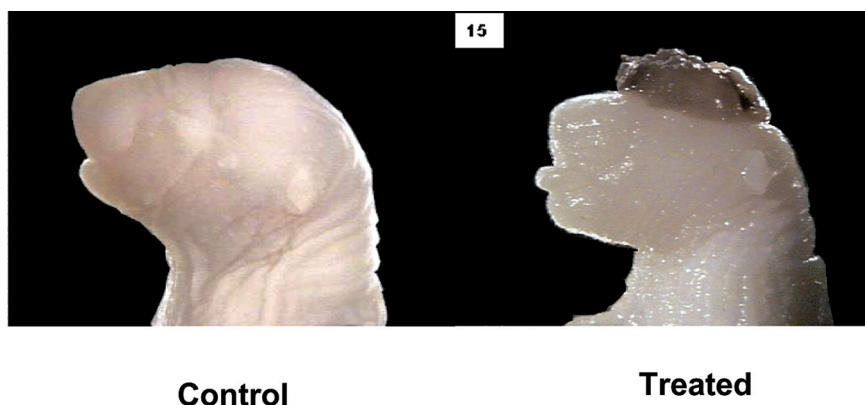


Figure 12.5 See text [page 544](#) for full discussion.

The Scope of Preclinical Drug Development: An Introduction and Framework

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The science of preclinical drug development is a risk-based exercise that extrapolates nonhuman safety and efficacy information to a potential human outcome. In fact, the preclinical development program for nearly every drug is an exercise in predicting clinical results with little data to support the use of the animal model under study. In the end, human studies validate the nonhuman models. Yet, understanding preclinical drug response—pharmacologic and toxic—with respect to dose, frequency, and route enables the clinical scientist to initiate and continue human trials under rational and ethical conditions. Those conditions include a starting dose and dose frequency that is at the highest region of a no effect level, a safe dose escalation scheme that permits differentiation of response as a function of drug exposure and an understanding of when potential toxicity may outweigh potential additional pharmacologic benefit (Fig. 1). Of similar significance but often under-appreciated is an understanding of pharmacokinetic and response variability—sources, type, and magnitude. So, while we report and often predict mean effect, there is rarely an average patient who will receive a drug. Consequently, most patients who receive a drug within a labeled dose regimen will not respond with an average pharmacologic response; nor, will most patients develop an average syndrome of adverse events. Rather, it is often vast diverse populations of individuals

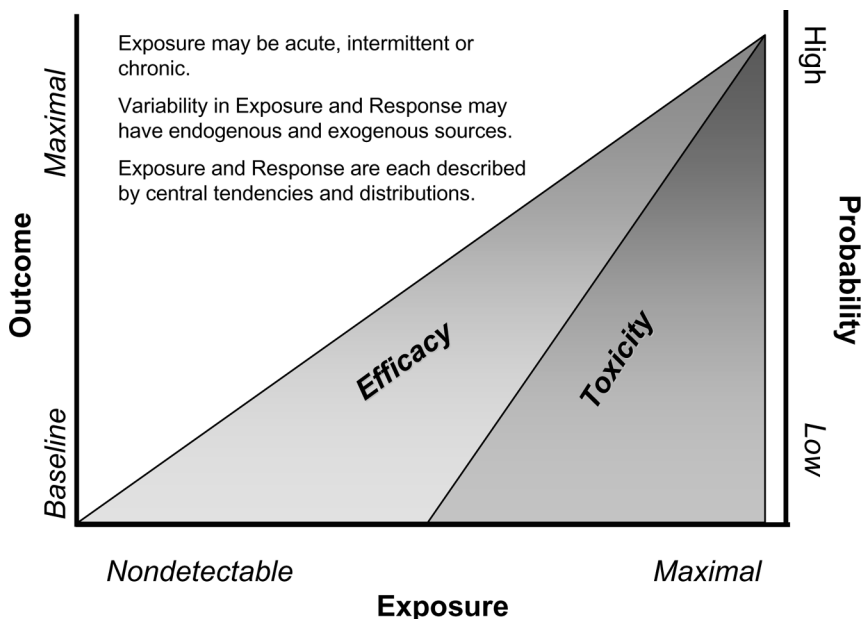


Figure 1 Exposure to drug product increases the likelihood of efficacy but also the potential for toxicity. For any given exposure, a probability distribution will exist for both efficacy and toxicity outcomes.

that receive pharmaceutical therapies. Phenotype, genotype, and lifestyle variability affect body composition and mass, drug transport and metabolism, and sensitivity to pharmacologic as well as toxic drug effects. Understanding sources of variability and the magnitude of that variability early in the development process permits clinical trial conduct that is most efficient and less likely to be encumbered with unanticipated events.

The realm of preclinical drug development can be compartmentalized into three disciplines that work in parallel from the stage of late research through clinical development. Two of these disciplines, pharmacokinetics and pharmacology/toxicology, are the subject of this text. The third discipline, bioanalytical research and development, is outside of the scope of this text and the reader will find many excellent publications elsewhere that are devoted to state of the art bioanalytical technology.

Before discussing the elements of a preclinical development program, some comments on the regulatory environment should be considered. The fundamental mandate of regulatory agencies is to ensure that clinical trials are conducted in a safe manner and that only drug candidates shown to be safe and effective are approved for commercial use. Early, scientifically rigorous interactions between a regulatory agency and industrial scientists will ensure that all concerns are addressed and that common objectives are determined.

The United States Food and Drug Administration operates an active program designed to understand and utilize preclinical models as predictors of human xenobiotic exposure. The organization conducting this research is the Division of Applied Pharmacology Research and it resides within the Office of Testing and Research (<http://www.fda.gov/cder/offices/otr/APRdefault.htm>). Members of this staff as well as members from the Office of Pharmaceutical Sciences generally serve as the FDA preclinical experts during a drug development program.

High-quality, efficient interactions with regulatory authorities will occur when the following conditions are met: (1) Understand regulatory requirements necessary to progress a drug candidate to the next stage of development. (2) Understand established—validated—technology that will be used in the drug candidate's development. (3) Understand state-of-the-art technology—not necessarily validated—that will be used in the drug candidate's development. While not always validated, that technology may offer substantial value to the development of a therapeutic candidate.

Preclinical drug development melds established and new technologies as a means toward predicting human outcomes. Established technologies can provide perspective relative to other drugs whereas nonestablished technologies can provide insight not otherwise available. While these latter technologies are a necessity it is imperative to respect their limitations (Fig. 2). Established technologies and study designs carry the value of being validated, generally well-controlled and having reference to historical databases. New technologies are typically not validated and, by their definition, do not have a relevant historical database for reference. Also, new technologies carry an inherent risk value. Certain new technologies may accurately and precisely measure a cellular event such as signal transduction, mRNA expression, or protein expression. However, until it is confirmed that these events robustly correlate with a therapeutic or toxic outcome, the technology carries high-risk value. Interpretation of data obtained from these technologies must be limited and not over-weighted when making decisions.

Nonetheless, the potential value of new technologies must be recognized and evaluation of these platforms must be embraced. Pharmacogenomics and toxicogenomics are poorly understood realms of science and each has considerable potential value. That value will materialize when these technologies develop validated standards and their output can be correlated with a reasonable probability to clinically relevant outcomes.

The International Congress on Harmonization has established a basic repertoire of guidelines that outline the technical requirements of acceptable preclinical drug development (www.ich.org). Also, the Center for Drug Evaluation and Research (CDER) has compiled a series of guidelines to assist the innovator with development issues; these guidelines may be found at the FDA website (www.fda.gov).

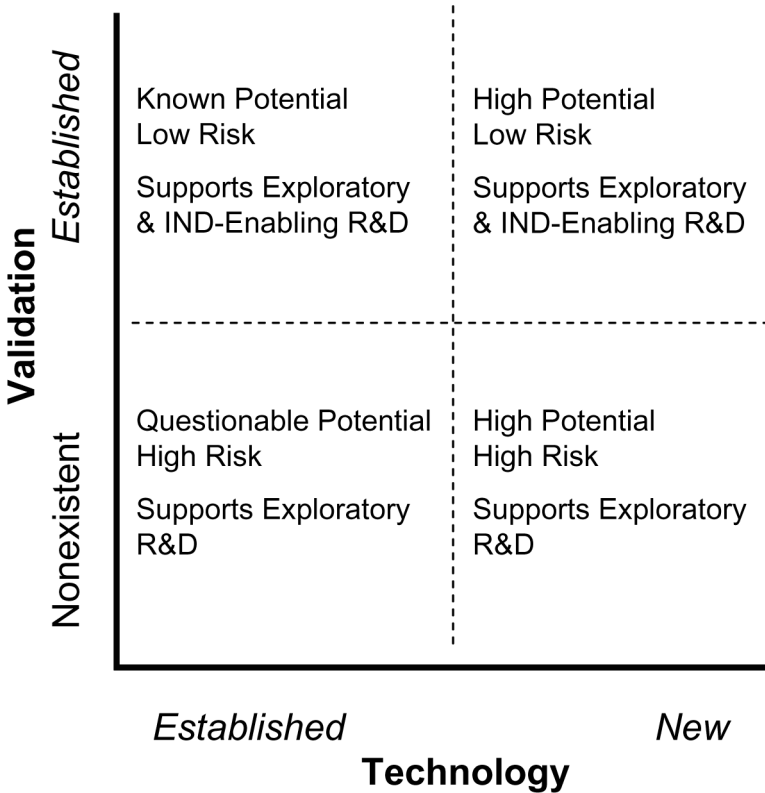


Figure 2 Novel therapies and next generation therapies are predicated on the use of established as well as new, but not yet validated, technologies. The inherent risk in new technology is unknown and may be high.

With the ongoing implementation of guidelines from the International Conference on Harmonization (ICH), the geographical regions of the United States, Europe and Japan have standardized approaches to the drug development process. However, while these guidelines provide a flexible and innovative basis for preclinical drug evaluation, they serve as a minimum requirement for achieving drug approval. It is generally accepted that additional studies may be required during the development process and that regulatory authorities amongst the three regions may have different scientific opinions on an acceptable preclinical development program.

The reader is cautioned that the ICH guidelines only constitute a minimum requirement and rarely encompass a development program that will be acceptable to the innovator company or all regulatory agencies. While the reader is encouraged to review and utilize these guidelines, a rational

preclinical information database must be predicated on minimizing clinical risk. The preclinical database strictly serves to predict (1) drug absorption and disposition and (2) the physiological outcome from exposure to that drug and its metabolites. This is very simply accomplished by conducting a series of studies that will form a foundation for efficient clinical development.

Figure 3 represents a temporal schematic of issues that are commonly addressed throughout the preclinical development program. In this example, the drug candidate might treat a chronic illness in a diverse patient population. A drug intended for acute or intermittent use or a drug intended for a narrower patient population might have fewer issues to consider and thus fewer studies in the program.

Figure 3 also illustrates that understanding the similarities and differences between nonhuman and human physiological systems is vital to obtain quality information from the program. Virtually every study and every decision to be made on the development of a drug candidate will be predicated on the assumption that preclinical models are a predictor of human exposure.

Shapiro addressed the issue of animal models that mimic human disease states and his thoughts can apply to the broader scope of this text (1). Quantitative validity of an animal model may have less value than the productive generativity of a model. While it is unlikely that anyone will ever validate a nonhuman model in a true or absolute sense, the nonhuman model will generate a body of evidence and confidence that the drug

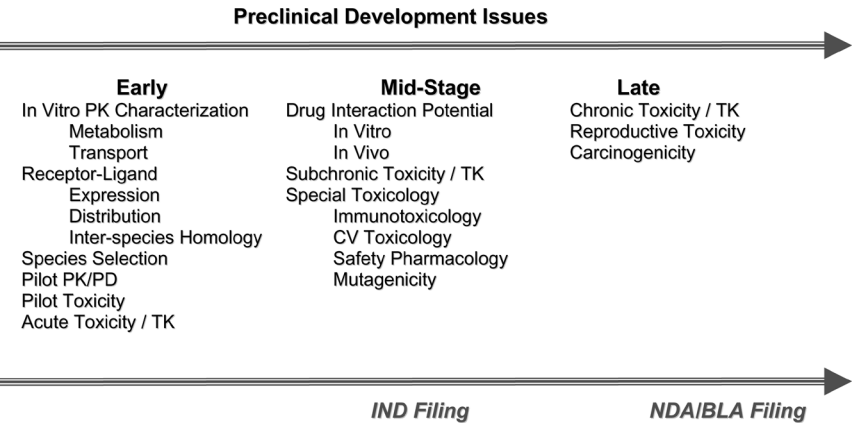


Figure 3 Preclinical development programs begin prior to IND-enabling work and extend through the clinical development stage. Each program is unique, is dependent on the intended therapeutic use, the potential patient population and historical reference. The following program might be acceptable for treatment of a chronic illness in a diverse population.

candidate is worthy of further development or should be terminated from the development pipeline.

A thorough understanding of any nonhuman model is fundamentally important so that drug-related outcomes can be separated from normal, endogenous variability or other processes unrelated to the drug. Rodents, canines, and nonhuman primates have become common preclinical models not always because of their strong direct relevance to potential human outcome but because of the established understanding of these animals and their underlying physiology (2–4).

In the following chapters, preclinical drug development will be reviewed in a sequence consistent with current rational and efficient practices. The reader will be introduced to animal models, species selection and will then proceed to chapters on definitive pharmacokinetic, pharmacodynamic and toxicology evaluations. Other important chapters describe formulation impacts, alternative technologies, and the relationship between preclinical findings and the clinical setting.

Looking into the future, the scientist who is engaged in preclinical drug development will more than ever factor innovation into the balance of risk vs. benefit (5,6). Even after rigorous preclinical and clinical evaluation, the potential for drug toxicity can be profound. For example, US drug R&D expenditures for 1995 were \$15.2 billion and had nearly doubled to \$30.5 billion in 2001. Yet, in the US alone over 100,000 patients die each year as a result of drug side effects (7,8). Furthermore, an additional two million patients require hospitalization or extension of existing hospitalization each year to treat drug side effects. While current preclinical safety assessments generally identify drug candidates with systematic toxicity potential, they remain insensitive to nonsystematic toxicity or to conditions that increase risk of known toxicity.

Weighing a drug's intrinsic toxicity relative to potential clinical benefit, those people conducting preclinical development become the messengers bearing safety margins and insight on early clinical development. There are limitations on how safe and efficacious a drug candidate can be made based on formulation, route of administration, and dose regimen. Hence, the most opportunity for achieving success lies with drug candidate selection. This is common sense but not often appreciated. Intelligent drug candidate selection incorporates knowledge of a molecule's (i) absorption, distribution, and metabolism properties, (ii) binding affinity to the intended pharmacologic receptor(s) and (iii) toxicity potential. Indeed, a 10-fold reduction in binding affinity may be more than offset by a bioavailability that has been improved by only 2–3 fold since increasing bioavailability reduces variability in absorption. For example, a drug with just 10% bioavailability has intrinsically poor absorption properties that may include poor solubility, dissolution rate, permeability or metabolic instability such as 1st pass metabolism. Consequently, dose-to-dose bioavailability may

range between 5% and 20% (and likely more). Inevitably this 4-fold fluctuation gives rise to subtherapeutic or toxic target tissue concentrations in some or all of the patient population and will likely lead to treatment failure. It is intuitive that variability in serum drug concentrations has less magnitude when absorption approaches 100%. In turn, it can be anticipated that as intrinsic bioavailability increases, the impact of food, age, and other factors on absorption will decrease. Clearly, in the quest for more potent and target-specific drugs, a similar effort must be exerted to achieve greatest bioavailability.

With respect to screening for drug clearance, numerous validated technologies are available to assess the potential for metabolism and likely routes of elimination (9–11). Greater utilization of human recombinant enzymes, cells, and tissues will accelerate our insight into appropriate selection of lead candidates for preclinical and clinical development. Likewise, isolated perfused organs can provide valuable insight into potential sites and mechanisms for drug metabolism and excretion.

Together, these technologies offer significant value in generating rank order information on lead drug candidates. In addition, they provide an early understanding of potential variables that may impact absorption or elimination.

With a lead drug candidate in hand an immediate understanding of drug disposition and elimination is demanded. Tissue accumulation, sequestration, and metabolism strongly influence the profile of pharmacologic effect and also give early warning on sites of potential toxicity.

Most promising in the advancement of pharmacokinetics and toxicology are the technologies that enable greater quantitative information to be gained on drug disposition and toxicity while using fewer animals. Advanced physiological-based pharmacokinetics (PBPK) and mixed effects modeling offer insight into drug disposition that can provide immediate value to the toxicologist and can also be extrapolated to potential human exposure (12,13).

Mahmood and Balian (14) and others (15) have published extensively on interspecies scaling techniques. The prediction of drug distribution volume, clearance, and half-life provides a rational basis for prospective preclinical and clinical study designs. While providing significant value to the development team these predictions also carry uncertainty and the scientist using the information must respect that caveat. Profound differences in anatomy and physiology between the preclinical species and humans can challenge the relevance of allometric scaling and, for that matter, all preclinical work. While rats have a lifespan that will not likely exceed 5 years, the lifespan of a human can often exceed 90 years. While rats have heart rates of ~360 beats per minute, the human heart rate is ~65 beats per minute. Respiratory metabolism, measured as O₂ consumption, is ~0.84 and 0.20 mL/hr/g in rats and humans, respectively. Similarly, there are also substantial differences in various organ blood flows, relative organ weights, and tissue architectures (16). Simple cross-species extrapolation of doses,

dose frequencies, or distribution of drug into tissues is likely to generate data with little predictive value.

In parallel, recent advances in identifying and quantifying gene expression and signaling processes permit mechanistic insight into drug activity and toxicity (17,18). Validation of new *in vitro* methods for toxicity assessment will further reduce animal use and increase the likelihood of a molecule entering clinical trials (19,20).

In summary, the understanding of preclinical drug disposition—distribution, metabolism, excretion—coupled with an understanding of cell or tissue specific activity/toxicity completes the knowledge base for a drug candidate to move into and through clinical evaluation. This understanding is achieved by generation of a clinical strategy that is then used to draft the initial preclinical plan.

Few, if any, preclinical plans remain intact throughout their lifespan. It should be anticipated that as studies are completed and observations are confirmed, ongoing and future studies are likely to require modification.

Throughout all development programs, it is imperative that the preclinical scientist assesses each study prior to implementation. What questions must be answered by the study? Do those questions warrant animal use or can more ethical *in vitro* methods be utilized. Does the proposed study have a high likelihood of answering those questions? If so, will the answers affect the subsequent clinical development? No study should ever be conducted unless there is clarity in the study goals and clarity in the expectations on how much risk is being eliminated from the clinical program by conducting the study.

The preclinical scientist is charged with advancing lead candidates into and through clinical development. This is achieved by reducing risk in the decision-making process for all clinic-related questions. While clinical risk is low, if not nonexistent, prior to human trials the cost of risk becomes substantial as the drug candidate moves into larger human trials and into more complex populations.

A scientifically sound preclinical program permits efficient, safe clinical development. The absence of such a program will promote poor decision-making and potentially serious clinical consequences. In this era of the public demanding better and safer medications both faster and at less cost, the preclinical scientist oversees a vital responsibility.

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Interspecies Differences in Physiology and Pharmacology: Extrapolating Preclinical Data to Human Populations

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1. OVERVIEW

Preclinical animal data are an integral component of the product development process, being used for predicting the potential for drug toxicity and for estimating first-time doses in humans. These extrapolations are based upon an assumption of a correlation between the exposure–response relationship in animals and man. Unfortunately, there is no single animal species that can serve as the “perfect” surrogate for human subjects, and the appropriate surrogate species needs to be evaluated for each situation (1).

Selection of the animal species to be used for toxicity testing should factor the potential interspecies differences that can influence systemic drug exposure and target cell sensitivity. These include potential differences in drug absorption, clearance, distribution, and metabolism. These factors can determine whether or not a species will exhibit toxicity or drug carcinogenicity (2).

In this chapter, the impact of study design, as well as interspecies differences in physiology and drug metabolism, will be explored from the perspective of the influence of these variables on the relationship between dose, drug exposure, and response.

2. TOXICOLOGY TESTS: POINTS TO CONSIDER

Several Offices within the FDA have Pre-Investigational New Drug Application Consultation Programs. These programs are designed to foster early communications between sponsors and the Agency. Advice may be requested for any aspect of drug development. All such communications are considered “informal” under 21CFR 10.90(b)(9) and do not obligate the Agency or sponsor.

As defined in CFR 312.23(a)(8), a sponsor filing an Investigational New Drug (IND) application must provide the results of pharmacological and toxicological studies of the drug involving laboratory animals and/or in vitro tests, which can provide the basis for the conclusion that it is reasonably safe to conduct the proposed clinical investigations. As drug development proceeds, the sponsor is required to submit informational amendments, as appropriate, with additional information pertinent to safety.

As per the regulation [CFR 312.23(a)(8)], the IND filing must include:

- i. **Pharmacology and Drug Disposition.** A section describing the pharmacological effects and mechanism(s) of action of the drug in animals, and information on the absorption, distribution, metabolism, and excretion of the drug, if known.
- ii. **Toxicology.** An integrated summary of the toxicological effects of the drug in animals and in vitro. Depending on the nature of the drug and the phase of the investigation, the description is to include the results of acute, subacute, and chronic toxicity tests; tests of the drug's effects on reproduction and the developing fetus; genetic toxicity testing; any special toxicity test related to the drug's particular mode of administration or conditions of use (e.g., inhalation, dermal, or ocular toxicology); and any in vitro studies intended to evaluate drug toxicity.

It is not unusual for the Agency to request draft/final study reports for the pivotal studies conducted in support of the initial IND, especially for new molecular entities.

Regulatory pharmacology and toxicology guidances involving animal models published by the International Committee on Harmonization (ICH) and/or the US Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER) include the following <http://www.fda.gov/cder/PharmTox/guidances.htm>:

Pharmacology:

- **Pharmacokinetics:** Guidance for Repeated Dose Tissue Distribution Studies; ICH-S3B (Mar 1995).
- **Toxicokinetics:** The Assessment of Systemic Exposure in Toxicity Studies; ICH-S3A (Mar 1995).

- Safety Pharmacology: Guidance for Industry: Safety Pharmacology Studies for Human Pharmaceuticals; ICH S7A (July 2001).

Toxicology:

- Single and Repeat Dose Toxicity: Nonclinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals; ICH-M3 (Jul 1997).
- Single Dose Acute Toxicity Testing for Pharmaceuticals; PT1 (Aug 1996).

Reproductive Toxicity:

- Detection of Toxicity to Reproduction for Medicinal Products; ICH-S5A (Sep 1994).
- Detection of Toxicity to Reproduction for Medicinal Products: Addendum on Toxicity to Male Fertility; ICH-S5B (Apr 1996).

Pediatric Drugs:

- Draft Guidance for Industry: Nonclinical Safety Evaluation of Pediatric Drug Products (Feb 2003).

Throughout the various ICH/CDER guidances, we see the same basic points to consider when selecting an appropriate animal model. These include:

- similarity in toxicological/pharmacodynamic responsiveness;
- pharmacokinetic profiles similar to those seen in humans;
- similar metabolic profile.

3. TOXICOLOGICAL ENDPOINTS

Observations generated in a toxicity study represent discrete protocol driven points on the dose effect profile. Therefore, the outputs from these tests serve only as experiment-wise approximations of the true continuous relationship between exposure and the biological effect. Nevertheless, these points serve as valuable information upon which to base first-time-in-human dosages of new chemical entities. Pivotal terms used to describe the results of toxicological investigations include (3):

NOEL—No Effect Level: The highest exposure level at which there is no drug-related adverse or non-adverse effect observed in the target population.

NOAEL—No Adverse Effect Level: The highest exposure level at which there are no statistically or biologically significant increases in the frequency or severity of an adverse effect between the exposed population and the corresponding control group.

LOAEL—Lowest Adverse Effect Level: The lowest exposure level at which there are statistically or biologically significant increases in the frequency or severity of adverse effects between the exposed vs. control populations.

MTD—Maximal Tolerated Dose or Minimally Tolerated Dose, depending on the implication. This is the dose at which biologically significant effects, directly/indirectly due to test compound administration, are found to have an appreciable effect on the quality and length of the lifespan of the animal. This may include a variety of outcomes, such as a lack of feed intake due to unpalatability of the test article, direct effect on the cardiovascular system, cecal dilatation and torsion due to changes in cecal flora in rodents.

Within this framework, an adverse effect is a biochemical, morphological, or physiological change that contributes to or is responsible for adversely affecting the performance (e.g., lifespan, health, well-being, growth) of the organism. Alternatively, it may reflect a reduced ability of the organism to respond to its environment. A biologically significant effect is a response that is considered to have a substantial or noteworthy positive or negative effect on the well being of the biological system. This is contrasted with a statistically significant effect that may not be meaningful to the state of health of the organism (3).

Some adverse reactions are also reflective of physiologic idiosyncrasies associated with a particular species. These do not correlate with exposure–response relationships in humans. Several of these peculiarities are summarized in [Table 1](#) (4).

In some cases, biological effects may reflect adaptive responses that are not related to the inherent toxicity of the test substance itself. An example of such a response is the histological change that may occur as an adaptive reaction to the inhalation of a compound (5). These include: mucus cell hyperplasia induced by dehydration of the nasal epithelium due to inhalation of aerosols; macrophage accumulation in the lung after exposure to low-solubility materials (in the absence of any other signs of an inflammatory reaction); and replacement of alveolar epithelium by ciliated epithelial cells as an adaptive response to high concentrations of exogenous materials.

Despite our best efforts, there will continue to be cases where toxicity in man could not be predicted from animal data. For example, fenclozic acid, which was a potential anti-inflammatory compound, was found to be without any adverse effects in an array of animal species including mouse, rat, dog, rhesus monkey, patas monkey, rabbit, guinea pig, ferret, cat, pig, cow, and horse. However, it caused acute cholestatic jaundice in people (6).

Table 1 Species-Specific Toxic Effects (4). Reprinted with Permission

Type of toxicity	Structure	Sensitive species	Mechanism of toxicity
Ocular	Retina	Dog	Zinc chelation
Ocular	Retina	Any with pigmented retinas	Melanin binding
Stimulated basal metabolism	Thyroid	Dog	Competition for plasma binding
Tubular necrosis	Kidney	Male rats	Androgen-enhanced sensitivity
Urolithiasis	Kidney and bladder	Rats and mice	Uricase inhibition
Teratogenesis: fetal mortality	Fetus	Rats and mice	Uricase inhibition
Cardiovascular	Heart	Rabbits	Sensitivity to microvascular constriction

4. FACTORS THAT CAN INFLUENCE STUDY RESULTS

Variables that can affect the outcome of studies intended to examine preclinical exposure–response relationships include the following (7).

- *Weight*: Animals of the same weight may have differences in lean tissue mass.
- *Age*: Age does affect sensitivity for some drugs in some species, including humans.
- *Sex*: Females of some species can exhibit more (or less) frequent toxic effects as compared to males.
- *Time of Administration*: Considerations include period of fasting, gastric emptying rate, and diurnal rhythms.
- *Temperament*: Stressors may cause a constriction of the splanchnic visceral blood vessels, which can affect drug metabolism and the proportion of the total cardiac output reaching the peripheral tissues.

Animal age is an important consideration when conducting toxicological studies to support drug use in human pediatric populations. The age of the animal used as the toxicological test species should be consistent with the intended age of the targeted human recipients due to potential differences in drug disposition and action, metabolism, body composition, receptor expression, and organ function that may occur in juveniles vs. adults. This issue is discussed in detail later in this chapter.

Formulation may also influence drug effects. For example, in mice, both the lethal dose (expressed as LD₅₀) and the ability to achieve some pharmacodynamic endpoint (e.g., righting reflex) for a fast acting compound (sodium pentobarbital) were significantly different when administered as an intraperitoneal injection of an aqueous solution or in a 1% carboxymethylcellulose solution. The decrease in pharmacological response with the 1% carboxymethylcellulose solution was attributed to an increase in product viscosity which, in turn, retarded drug uptake (8). This simple example underscores the influence of formulation in preclinical studies. Additional insight into potential species-specific considerations in drug formulations have been published elsewhere (9).

Excipients used in preclinical drug formulations can markedly affect the level of drug exposure. Permeability enhancers such as the bile salt sodium deoxycholate (10), fatty acids such as sodium caprate (10), and surfactants (11) such as polysorbate 80 (12), Cremophor EL (13), and vitamin E (14) can alter P-glycoprotein (P-gp) activity. P-gp is a membrane transporter protein that can affect the first-pass drug loss of many compounds. The role of P-gp in determining drug oral bioavailability is discussed later in this chapter and in [Chapter 8](#).

Differences have been observed in the ability of the various animal species to express toxic reactions similar to that in humans (15). In a survey of the 20 chemical entities for which preclinical and clinical toxicity information was available, monkeys, rats, and mice appear to exhibit the greatest similarity to humans in adverse events. Dogs are associated with a more frequent occurrence of false-positive reactions (Table 2).

Similarly, in comparing the accuracy of the predictions of human drug toxicity generated in dogs and monkeys, Schein et al. (16) observed that monkeys and dogs tend to correctly predict bone marrow depression, gastrointestinal disturbances, and hepatotoxicity. However, these same species present with a high percentage of false positives. Of the 25 anticancer drugs investigated, dogs exhibited a particularly high rate of false positives for pathology of the stomach, small and large intestine, liver (including increases in alkaline phosphatase) and kidney (including proteinuria).

Table 2 Correlation of Toxicity to that Observed in Humans (15). Reprinted with Permission

	Rat	Mouse	Dog	Monkey
# Comparisons with humans	14	11	11	6
Similar to human ($\pm$)	71%	73%	45%	83%
False-positive	21%	—	36%	—
False-negative	7%	27%	18%	17%

The rate of false positives in monkeys was slightly less than that of dogs. Cases where neither species expressed toxic reactions seen in humans were rare, although examples of renal, cardiovascular, and neuromuscular toxicity do exist.

With regard to rats, basic human–rat differences in physiology may affect study outcome. Unlike humans, rats can synthesize ascorbic acid, have no gall bladder, are coprophagous, are obligate nose breathers, and have important differences in their lung function and morphology (17). Moreover, when rodents are treated with antimicrobial agents, they frequently develop cecal dilation and torsion due to alterations in their intestinal flora. This finding may preclude their use as models for development of these drugs. Monroe and Mordenti (17) have summarized the physiological, anatomical, and biochemical factors that can be considered in preclinical studies when analyzing data from studies that employ rats as the target species. Their summary is reproduced in [Table 3](#).

Strain of animal may also affect study outcome. For example, there is greater tobramycin toxicity observed in Fischer rats as compared to Sprague–Dawley rats (18). Differences in toxic responses between species of monkeys are also evident. Stump-tailed macaques exhibit the same thrombocytopenia as that seen in humans with compound BL-4162. However, neither the rhesus monkey, cynomolgus monkey, squirrel monkey, nor the chimpanzee exhibit that same toxic effect (15).

Conditions associated with animal care, such as crowding, isolation, temperature, food or water restriction, alteration of light–dark cycle, immobilization, handling, and drug administration procedures, can result in physiological changes that are not drug related. Each of these conditions can alter the release of hormones such as adrenal corticotrophic hormone, thyroid hormone, insulin, and many of the pituitary hormones. In turn, the latter can modify responses to the various toxicants (4,19).

The dose–effect relationship can also be influenced by normal diurnal rhythms. For example, both hepatic and renal functions exhibit diurnal variation in mice. Metabolism is higher during the active dark phase as compared to the light phase (20). Significant circadian-related fluctuations in drug pharmacokinetics have been observed for a wide variety of drugs including antimicrobial compounds, neurological and psychiatric drugs, anti-inflammatory drugs and cardiovascular agents (21). There can be marked diurnal variability in disease expression and drug therapeutic activity (22–24). Similarly, the magnitude of drug toxicity may vary as a function of administration time (25–27). In some cases, circadian variability in drug toxicity has been attributable to fluctuations in the activity of certain metabolic pathways (27), and these variations may not be equally expressed in males and females (28).

Fasting can alter drug pharmacokinetics. In addition to the relationship between prandial state and such factors as gastric emptying, drug

Table 3 Factors that may Influence the Ability to Extrapolate Toxicity and Carcinogenicity Data from Rats to Humans (based upon Table by Monroe and Mordenti (17))

Parameter	Rat	Human	Comment
Body weight (kg)	0.35	70	Humans have hundreds more cellular (DNA) targets for carcinogenic attack
Surface area (m ²)	0.05	1.75	
Lifespan (hr)	2.5	70	Humans can be exposed much longer, but the aging and carcinogenesis processes are interrelated
Food consumption (dry) g/kg BW/day	50	10	High intake of lipid and protein leads to cumulative oxidative damage that contributes to aging and cancer
Basal metabolism (kcal/kg/day)	109	26	High metabolic rate correlates with DNA oxidative damage
Anatomical			
Forestomach, Zymbal's gland, Harderian gland, preputial gland, clitoral gland	Present	Absent or rudimentary	Difficult to interpret tumors in organs present in one species but not the other
Bronchial glands	Absent	Present	
Emetic reflex	Absent	Present	May retain some toxicant that humans would not
Liver weight (% body weight)	5%	2.20%	Rates of organ growth and cell turnover may contribute to carcinogenic susceptibility
Physiological			
Reproductive cycle	Estrus	Menstrual	Different patterns and roles for estrogen and progesterone may affect susceptibility to certain cancers.
Parity	High	Low	Pregnancy protects against some cancers

Prolactin—role in mammary gland activity α -2 μ -globulin	High	Questionable	Modulations of prolactin secretion will have different consequences for mammary carcinogenesis Protein necessary for some renal and perhaps bladder cancers
Stomach pH	Present, especially in males 4–5	1–2	Can affect activation/deactivation of some xenobiotics that undergo enterohepatic cycling
Bacterial flora Thermoregulation	Numerous	Few	Key controlling tissue in rats is brown fat; in human it is the dermal vasculature
Hematology (expressed relative to human values)			
GSH-peroxidase	10.2	1	Enzymes important in countering oxidative damage to cells
GSH reductase	0.2	1	
Superoxide dismutase	1.7	1	
DNA repair			
Excision repair	Low		May be a factor in determining lifespan and in defense against DNA alterations
Hepatic O ⁶ -alkyl guanine transferase	1	10	Key enzyme in detoxifying a common type of DNA–carcinogen adduct
Xenobiotic metabolism			
Epoxide hydrolase (liver)	Low	High	Enzyme important in detoxifying epoxides
Phase I and II enzymes			Difficult to predict transformation pathways in the two species

dissolution, enterocyte permeability, and hepatic blood flow (29,30), fasting itself can significantly affect the level of several metabolizing enzymes. In some cases, cycles in eating activity are responsible for the apparent circadian variability in drug pharmacokinetics (31). Partial dietary restriction was found to exert a protective effect against certain types of carcinogens. When fed 75% of ad libitum intake, rats were found to have a significant reduction in certain types of tumors (i.e., pituitary adenomas, hepatic foci) as compared to animals provided food ad libitum (32).

The composition of the animal diet can also influence the response to toxic agents. Rats fed diets deficient in vitamin A or beta carotene produced significantly higher rates of malignant tumor formation in response to exposure to aflatoxin B1. However, diets containing 10 times the normal levels of vitamin A did not result in a protective effect above that observed under control conditions (32).

Dietary fats themselves may affect drug pharmacokinetics (33,34). This variable may influence the results of toxicological studies since prior to oral administration in rats, lipophilic compounds are frequently dissolved in dietary vehicles such as corn oil, olive oil, or sesame oil. While these vehicles do not appear to significantly alter drug metabolism when administered in an amount consistent with that used during experimental dosing, significant changes were found to occur in the levels of certain microsomal enzymes (e.g., increased CYP 3A and decreased CYP 2C11). Accordingly, the possibility that these dietary oils may influence hepatic CYP-mediated drug metabolism or exacerbate certain CYP-mediated drug-drug interactions cannot be discounted (34).

In response to concerns regarding the influence of dietary fats on the outcome of toxicological studies when used as gavage vehicles, the National Institutes of Health sponsored a study comparing the toxic effects of corn oil, safflower oil, and tricaprylin (35). Each gavage dose was administered at volumes of 2.5, 5, or 10 mL/kg daily for 5 days per week for a total of 2 years. Observed effects of these oils included hyperplasia and adenoma of the exocrine pancreas, a decrease in the incidence of mononuclear cell leukemia, and a reduction in the incidence or severity of nephropathology in male rats. There was also an increase in the incidence of squamous cell papillomas of the forestomach of rats receiving 10-mL tricaprylin/kg. For the most part, this investigation demonstrated that all three oils were capable of causing dose-related toxicities, and that it is the level of fat rather than the degree of saturation that is the most important consideration in this regard.

In a two-year study where a 500 mg total dose of dichloromethane was administered in corn oil (2.5, 5, or 10 mL/kg) to male rats, the use of a corn oil vehicle substantially reduced the highly toxic effects associated with dichloromethane. When administered without corn oil, the dichloromethane group exhibited severe toxic reactions. However, rats survived the 2-year

study period when administered this compound in corn oil. Pathological findings were consistent both with the dose-related toxic and protective effects of the corn oil itself, as well as toxic effects of the dichloromethane (35). While this investigation substantiated that oily vehicles can influence the results of a toxicity study, a note of caution was raised with regard to the interpretation of some published investigations. In particular, several reported relationships between oil intake and carcinogenicity were found to use control groups administered diets deficient in vitamins, essential amino acids, or energy. Such deficiencies can inhibit the growth of neoplasms.

Sex differences themselves can be species specific. For example, in male rats, the rate of microsomal metabolism tends to be higher than that in females (36). This may lead to sex-related differences in the level of the parent compound or an active metabolite. In evaluating 98 pesticides, Gaines (37) observed that the majority of orally administered drugs were more toxic in females as compared to males. The reverse was true for only 9/98 compounds. However, similar sex-related differences were not observed in dogs (38). Studies involving other P450 systems likewise support the premise that rats tend to express sex-related differences more frequently than do other animal species (38,39). In part, sex differences in the daily rhythm of rat hepatic enzymes have been linked to sex differences in the pattern of growth hormone secretion (28).

In humans, there is a statistically significantly higher level of plasma cholinesterase in young healthy males as compared to females, where the activity in females is estimated to equal 64–74% that of males. This difference disappears in geriatric individuals (40). In contrast, there are no significant sex-related differences in erythrocyte cholinesterase (41) or in brain cholinesterase (42). Therefore, toxic effects associated with anticholinesterase agents in humans may or may not exhibit sex-related dependencies, depending upon the age of the recipient, the drug's site of action, and its relative affinities for the various forms of cholinesterase.

Although this chapter focuses on exposure to xenobiotics via the oral or parenteral routes, it is important to note that additional concerns may arise when examining exposure by other routes such as inhalation and dermal (4). For example, while rabbits are often used for testing the toxicity of dermal exposure, it is the pig that most closely resembles the dermal absorption characteristics of humans. With regard to inhalation exposure, the following differences need to be considered when extrapolating between rats and humans:

- There is a significant difference in the filtration size of particles that are inhaled via the nose (3 μ filtration) vs. that by mouth (10 μ filtration). This will impact the nature of particulate drug exposure in

humans (which are nose and mouth breathers) vs. rats (which are obligate nose breathers).

- The number of daughter generations of the air passage in humans is 35, while there are fewer than 25 generations in the rat.
- The total lung volume of rats is only 10% that of humans.

Furthermore, in contrast to convention, the species most closely resembling humans with regard to respiratory system structure and function are the horse and donkey.

In general, when using multiple species for assessing the risk of drug toxicity in humans, the probability of an inappropriate conclusion is generally low (43). Boxenbaum and DiLea (44) estimated these risks in an effort to predict the likelihood of a serious adverse event when a drug is administered as a first-time dose to healthy human subjects:

- Sum of observed occasions when rat and other non-primate species exhibited a “good” or “fair” model for human drug toxicity = 0.92.
- Frequency of an adverse event that is predicted incorrectly = 8% of total tests. This may be attributable to an adverse reaction seen in animals that does not occur in humans, or an adverse event in humans that was not predicted in animal studies.
- Assuming a 5% risk of failure to predict an adverse event in humans and given the safety factors built into the estimate of the first-time dose in humans, of that 5% risk, only 1% of those events is serious. Accordingly, the risk of a serious adverse event associated with studies involving first-time dose in humans = $0.05 \times 0.01 = 0.0005$. In other words, in only 0.05% of the times do we anticipate that an unexpected serious adverse event will occur when a drug is administered for the first time to human subjects when there is an appropriate and adequate preclinical toxicity profile on which the assumptions are based.

During a workshop of the International Life Science Institute in which the toxicity of pharmaceuticals in humans and laboratory animals was compared (45), it was concluded that an interspecies difference in parent drug exposure was an unlikely cause for differences in adverse reactions. Rather, interspecies differences in target tissue response and drug metabolism were concluded to be the more likely reason for many of these discrepancies.

5. INTERSPECIES PHARMACOKINETIC DIFFERENCES

5.1. Drug Absorption

A host of physiological variables may contribute to interspecies differences in drug absorption and bioavailability. These variables include drug product

dissolution, gastric transit time, intestinal permeability, first-pass drug loss, and food effects. Interspecies differences in gastrointestinal physiology and the impact of these differences on drug absorption have been reviewed elsewhere (9,29).

Much of the interspecies' diversity in gastrointestinal (GI) anatomy and function reflects differences in primary sources of dietary constituents (46–49). For example, carnivores (e.g., dogs, cats) possess a relatively simple colon but a well-developed small intestine (long villi). This is consistent with a diet that is low in fiber, but high in fat and protein. Omnivores (e.g., rats, pigs) possess a well-developed small intestine but have a more complex lower intestine to compensate for their more diverse diet. The lower intestine of pigs is differentiated enough to allow for dietary fiber fermentation. Herbivores are either foregut (e.g., sheep) or hindgut fermenters (e.g., horse) and rely upon fermentation processes for nutritional intake. Intestinal villi vary in length from 0.5–1.0 mm, depending on region and species. They are generally long and slender in carnivores, and short and wide in ruminants. Rats, mice, and horses lack an emetic reflex (6,48), and rats and horses lack a gallbladder (6).

The digestive systems of ruminants differ markedly from those of monogastric species, and these differences can significantly alter drug absorption (48). In the case of ruminants, the forestomach (rumen, reticulum, and omasum) is a large volume compartment lined with stratified squamous epithelium. This site of microbial fermentation will both catabolize cellulose-containing materials and degrade drugs. The capacity of this is 10–24 L in sheep and goats. The pH values range from 5.5 to 6.5 due to a large volume of alkaline saliva (pH 8–8.4) that is secreted to buffer organic acid production in the rumen. Although gastric juices are not secreted in the forestomach, the rumen has a large capacity for drug absorption, particularly for weak acids. The abomasum, the fourth chamber, is the true stomach and secretes digestive juices. The generally larger hepatic capacity of herbivores tends to result in greater metabolism of lipophilic compounds.

Interspecies differences in GI transit time can markedly affect the extent of drug absorption and consequently, dose–effect relationships. An illustration of this is the failure of beagle dogs to adequately model the human bioavailability of acetaminophen sustained release tablets (50), griseofulvin tablets (51), valproic acid (52), and ampicillin (53). When comparing the gastric emptying of fasted humans, dogs and minipigs, the order in rate of gastric emptying is dogs > humans > minipigs (54). These differences are observed both with tablets (enteric-coated aspirin, diameter 5.8 mm, 1.24 g/cm³ and barium sulfate tablets, 6.0 mm diameter, 1.52 g/cm³) and granules (diameters = 0.1 mm, density = 1.17 and 1.34 g/cm³, respectively). Tablets empty more rapidly than granules in dogs, but are cleared at a similar rate in humans. In contrast, granules tend to clear

slightly faster than tablets in pigs, as evidenced by the time to 75% remaining in the stomach.

Despite the faster gastric emptying observed in dogs vs. humans under fasted conditions, food induces a substantially greater delay in the emptying of large particles (tablets) and pellets in dogs as compared to humans (55). For example, in dogs, gastric emptying of pyridoxal phosphate enteric-coated tablets continued to occur for more than 10 hr in fed dogs. In contrast, gastric emptying of these tablets in humans did not extend beyond 5-hrs after postprandial administration.

Marked interspecies differences are also observed in intestinal transit times. When fluid or particulate markers were administered intragastrically, the percent of dose excreted in the feces from hours 0 to 24 in dogs vs. mature swine are 55% and 7%, respectively for the fluid markers. For particulate markers, 24 hr fecal excretion was 40% and 2% in dogs and swine, respectively (56). Sustained release preparations (eroding matrix) of the lipophilic compound, propylthiouracil, demonstrate very poor bioavailability in dogs because the rapid GI transit does not provide the time needed for complete product dissolution. Generally, the product will reach the canine colon (2–3 hr) before having had an opportunity to dissolve (57).

Depending upon the pK_a of the drug in question, differences between human and canine gastric pH can lead to differences in the extent of drug dissolution. Accordingly, interspecies deviations in gastric pH have been implicated as a cause for dissimilarities in the bioavailability of indomethacin (58), metronidazole (59), and cinnarizine (60). However, differences in gastric pH are confounded by further interspecies differences in the food effects. Generally, the gastric pH of fasted dogs is highly variable, ranging between 3 and 8 (61). Following a meal, gastric acid secretion rates in dogs exceed those of humans and swine. The postprandial gut pH in humans tends to exceed that observed in dogs due to the strong buffering action of the diet, but human gastric pH values return to baseline values within approximately one-hour (62).

Interspecies variation in intestinal absorptive surfaces can result from dissimilarity in the size and shape of the intestinal villi (62). Differences in surface area for paracellular absorption could influence the relative bioavailability of small hydrophilic (low permeability, high solubility) compounds. Conversely, highly permeable drugs are generally absorbed upon contact with the intestinal membrane, with the majority of absorption occurring at the villus tip (63). Minimal (if any) differences in the absorption of highly permeable compounds across animal species are anticipated.

To date, little information is available with regard to interspecies differences in lymphatic uptake. In part, this may be due to bias associated with the types of methods used to assess lymphatic drug uptake in animals (64). Nevertheless, the comparative extent of drug uptake into the lymphatic system across species may be expected to be influenced both by the

characteristics of lymph flow to various absorption sites and by the mechanism through which the lymphatic absorption occurs. Since lipid digestion may be expected to differ between herbivores and carnivores, it would be reasonable to expect better lymphatic uptake in carnivores or omnivores as compared to herbivores. This may also be attributable to diet-related differences in bile salt composition and its corresponding impact on lipid solubilization (65). Furthermore, since the density of intestinal lymphoid tissue shows species-related differences, we may expect dissimilarities in lymphatic entry into specialized tissues such as Peyer's Patches (66).

Comparative estimates of oral bioavailability are often linked to species-specific differences in drug metabolism occurring in the gut and/or the liver. The principle intestinal biotransformation enzymes in humans include the cytochrome P450 (CYP) subfamily, glucosyltransferases, sulfo-transferases, *N*-acetyltransferase, glutathione *S*-transferase, esterases, epoxide hydrolase and alcohol dehydrogenase (67). Within the gut wall, differences in site-specific drug metabolism are known to occur across animal species. For example, esterase activity, while present in the order of duodenum > jejunum > ileum > colon, is greater in rats as compared to pigs and man. The esterase activity of humans is somewhat greater than that of swine (68).

The relationship between drug absorption, gut metabolism, liver metabolism, and drug bioavailability is described by the following relationship (69):

$$F = f_{\text{abs}} \times (1 - f_{\text{g}}) \times (1 - f_{\text{h}})$$

where F is the absolute bioavailability of the drug, f_{abs} the fraction of the dose absorbed from the GI lumen, f_{g} the fraction of drug metabolized by the gut wall and f_{h} the fraction of drug metabolized by the liver.

Since the permeability of molecules across the gut wall tends to be similar across species, the predominant cause of dissimilar bioavailability across animal species is related to corresponding values of f_{g} and f_{h} .

The importance of first-pass metabolism is seen with indinavir. Differences in oral bioavailability (72% in dogs, 24% in rats, and 19% in monkeys) are attributable to species-specific variations in the extent of hepatic first-pass extraction (approximately 68% in rats, 65% in monkeys, and 17% in dogs) (70). In human subjects, the oral bioavailability of indinavir is approximately 60% (71). In a survey conducted by Chiou and Barve (72) and Chiou et al. (73), the oral bioavailability of most drugs tended to be substantially lower in dogs as compared to rats and humans, due largely to the greater first-pass drug loss seen in dogs. In contrast, drug bioavailability in rats and humans tends to be highly correlated.

The small intestine is a potential site of drug metabolism, and substantial drug loss can occur via intestinal efflux mechanisms, gut wall

metabolism (both Phase I and Phase II), and degradation within the gut lumen (69,74,75). While the total amount of P450 in the human intestine is much less than that in the liver (20 pmol/mg microsomal protein, vs. 300 pmol/mg microsomal protein, respectively), the intestinal enzymes are strategically situated to maximize exposure to the intestinal contents. P450 concentrations tend to be greatest in the villus tips of the upper and middle third of the intestine (76).

Of particular importance is the synergy between P-glycoprotein (P-gp) and CYP 3A4, which together are responsible for the active extrusion and subsequent metabolism of a wide variety of compounds (77). P-gp is located on the apical surfaces of many organs including the bladder, kidney, brain, liver, lungs, pancreas, stomach, spleen, esophagus, and the large and small intestines (78,79), and interspecies differences in the tissue of expression of drug transporters have been observed (80). In the intestine, the ratio of fluxes from basolateral to apical vs. apical to basolateral direction ranges from 1.4 to 19.8, depending upon location within the GI tract (81). Evidence suggests that P-gp substrate affinity may vary as a function of intestinal site (82).

The importance of P-gp may be most clearly seen in bioavailability studies conducted with mice expressing the *mdr1a* $-/-$ genotype ("Mdr1a knockout mice"). This strain exhibits a total absence of gut P-gp activity. These mice were used to examine P-gp role in limiting the intestinal absorption of paclitaxel (83). Paclitaxel AUC values after oral administration in wild-type mice (*mdr1a*+/+) vs. knockout mice (*mdr1a*-/-) were 11% and 35%, respectively. Intestinal secretion following intravenous administration was practically eliminated in knockout mice, even though 40% of the dose underwent intestinal secretion in the wild-type mice. Similar differences in wild-type vs. knockout mice bioavailability were observed for compounds such as vinblastine, digoxin, indinavir, and talinolol (84). An effect corresponding to the *mdr1a* $-/-$ genetic variant of mice was observed in humans, where certain variations in the *Mdr1* gene have been shown to alter both the gut expression of P-gp and the oral bioavailability of P-gp substrates (85).

Compounds may also be extensively metabolized in the gut lumen by digestive enzymes or by activity of the gut microflora. The colon contains the largest populations of microorganisms in the monogastric GI tract and is the major site of production and absorption of volatile fatty acids in the pig, rabbit, rat, dog, and human (62). An excellent example of the potential negative impact of microbial metabolism is the species-by-route differences in blood concentrations achieved when chloramphenicol is administered to goats, pigs, dogs, cats, and horses. Despite high levels achieved in the goat after intramuscular administration, the oral bioavailability of this compound in goats was negligible due to microbial degradation in the gut. Similar problems did not occur when this compound was orally

administered to monogastric species (86). Conversely, the presence of gut microflora may enhance drug bioavailability by promoting biliary recycling of compounds such as ouabain, digoxin, and steroid hormones (e.g., 87). In these cases, the bacteria remove the polar moiety from the derivatized conjugates, rendering them available for intestinal absorption (74).

In contrast to the oxidative and conjugative metabolism of the liver and intestinal mucosa, bacterial metabolic reactions are largely degradative, hydrolytic, and reductive. As such, they are involved in the enterohepatic recirculation of many compounds. Drugs conjugated with polar groups in the liver prior to their secretion into the bile are hydrolyzed within the upper and lower intestine. Beta glucuronidase, sulfatase, and glycosidases are all bacterial enzymes found in the gut of human and domestic animal species (88,89).

An example of how bacterial flora can impact drug toxicity is seen with chenodeoxycholate (CDCA), a compound used to facilitate the dissolution of gallstones in man. It was found to produce toxic effects in rats, hamsters, rabbits, dogs, rhesus monkeys, and baboons, but found not to be toxic to the squirrel monkey, chimpanzee, or humans (90,91). This species-specific sensitivity has been correlated with the ability of their respective intestinal flora to produce a toxic (sulfated) metabolite of CDCA.

5.2. Drug Metabolism

Drug metabolism can be considered from the perspective of its influence on systemic exposure to the parent compound (i.e., clearance processes) or on the formation of potentially toxic metabolites. Accordingly, confounding the interpretation of *in vivo* toxicity study data are both the qualitative and quantitative interspecies differences in drug metabolism. Such differences are not uncommon, and an understanding of these factors can contribute to the interpretation of toxicity study data (92).

Benzidine is an example of where interspecies differences in drug metabolism lead to species-specific toxic reactions (93). In dogs, hepatic *N*1-glucuronidation of benzidine forms an acid-labile conjugate that is transported in the blood while bound to plasma proteins. Upon being filtered by the kidney, the drug accumulates in the urine whereupon acid hydrolysis releases the amine. The amine is subsequently activated by bladder enzymes, thereby initiating the carcinogenic process. In rats, liver rather than bladder cancer is the endpoint, presumably due to the low capacity of rat liver UGT to conjugate the benzidine.

The Laboratory of Clinical Pharmacology of the U.S. Food and Drug Administration (FDA) provided other examples demonstrating the importance of understanding interspecies differences in drug metabolism when assessing preclinical study data (94):

- Paclitaxel is used in a polytherapy regimen. This may include its use in combination with other anticancer drugs or its co-administration with agents intended to minimize allergic reactions. In humans, the primary mechanism of drug elimination is via CYP2C8. However, negligible amounts of this enzyme are present in rat microsomes. Therefore, rats cannot be used for examining drug–drug interactions in humans. In contrast, since paclitaxel itself is the primary agent of interest from both a toxicological and effectiveness perspective, the rat is an appropriate model for toxicity studies.
- Due to the rapid glucuronidation of zidovudine in humans (70–80%), the terminal elimination half-life in humans was much shorter than that expected based upon animal model data (dogs and rats). To maintain efficacious levels in humans, the frequency of dosing needed to be increased from bid, which was predicted on the basis of animal studies, to q4 hr.
- Iododeoxydoxorubicin is a drug for which there exists large quantitative interspecies differences in drug metabolism. This renders preclinical study data to be of questionable relevance to humans. While in rats, the parent drug is the predominant circulating moiety, in humans, there is a 10-fold greater exposure to metabolites as compared to the parent compound.

Variations in biotransformation generally occur in one of three forms (95):

- Species-specific deficiency in a particular metabolic reaction.
- Species-specific limitations in particular metabolic reactions.
- Variations in the activities of competing metabolic reactions.

When similar enzymes are involved in drug elimination, the (weight adjusted) intrinsic clearance of the compound generally tends to be greater in the smaller as compared to the larger mammalian species (96). However, exceptions to this pattern have been observed (97).

Generally, metabolic processes are classified as either Phase I or Phase II reactions. Phase I reactions are typically oxidative and add or expose polar functional groups on a lipophilic substrate. Phase II metabolic reactions are typically conjugative, reacting molecular functional groups (be it associated with the parent compound or a product of Phase I metabolism) with an endogenous substrate to yield a metabolite that is readily excreted. Generally, the Phase II metabolites are inactive, although certain compound classes, such as the reactive acyl glucuronides of xenobiotic carboxylic acids, do present with clinically relevant toxicities (92). Whether Phase I metabolites result in toxicity or detoxification may depend upon the presence or absence of subsequent Phase II metabolism.

Certain metabolic reactions appear to be negligible or even totally lacking in certain animal species. Examples include (95,98):

- Rat: deficiency in the *N*-hydroxylation of aliphatic amines.
- Dog: inability to acetylate compounds.
- Guinea pig: deficiency in *N*-acetylation and unable to *N*-acetylate *S*-substituted cysteines.
- Cat: deficiency of glucuronidation reactions.
- Pig: deficiency in most sulfation reactions.

In other cases, there are drug-specific metabolic reactions that appear to occur in only certain animal species. An example includes the *N*-glucuronidation of sulfadimethoxine and other methoxysulfonamides, which appear to be limited to man and certain primates (95).

Numerous examples of metabolic divergence across animal species have been reported. Intestinal Phase II biotransformation activities, which are carried out by UDP glycosyltransferases (UGT) and sulfotransferases, are found to be higher in the rabbit than in the rat (99). Cultured hepatocytes from goats, sheep, cattle, and rats show similarities in glucuronidation and sulfation. However, while the enzymatic activities associated within goat liver cells showed higher activity in females vs. males, the opposite gender effect was observed in rats (100). Metabolic idiosyncrasies also can be correlated with animal diet: herbivores tend to be far more efficient than other species with regard to oxidative reactions (101).

In the case of the β -blocker, acebutolol, this drug is hydrolyzed to an aromatic amine in man and then subsequently acetylated to the active metabolite, diacetolol. The latter not only has very potent antihypertensive activity but also exhibits a markedly longer elimination half-life (8–13 hr) as compared to acebutolol (3–4 hr). In contrast, dogs are unable to form diacetolol because of their deficiency of the enzyme arylamine acetyltransferase. Accordingly, markedly different pharmacological activities and toxicological profiles can be expected in dogs vs. humans (102).

When the metabolite profiles are qualitatively similar across species, what factors can lead either to differences in the intrinsic clearance of that compound or to differences in drug–drug interactions? Proposed factors to consider include (97):

- A metabolic pathway may be catalyzed by different enzyme isoforms in different species.
- Different inhibitory sites may be present, even if the same enzyme subfamily is involved in that drug's metabolism.
- Species differences in enzyme-specific ratios may lead to variability in the activity of metabolic inhibitors. For example, the ratio of

CYP1A2 and CYP1A1 is 4–20:1 in most mammalian species, but is 0.14–0.67:1 in the rat.

- Slight differences in the enzyme's amino acid sequence may lead to marked differences in substrate specificity and enzyme activity.

Of particular interest is the cytochrome P450 family (particularly CYP1A1 and 1A2) since these are implicated in the carcinogenic activation of numerous xenobiotics (103). In man, the three major forms of cytochrome P450 (CYP) are CYP2D6, CYP2C9, and CYP3A4. While CYP1A2, CYP2C19, and CYP2E1 are also important, their involvement tends to be far less extensive than that associated with the former three isoforms (104).

Caffeine is often used as a metabolic probe for the activity of this family of enzymes. Using hepatic microsomes from humans, monkeys (*Macaca fascicularis*), rats, rabbits and mice, three dimethylxanthines were formed, resulting from *N*-demethylation (theobromine, paraxanthine, and theophylline) and one compound resulting from oxidation at the C-8 position (trimethyloric acid) (105). Despite qualitative similarities, the relative proportion of the metabolites was markedly different across animal species. The ratio of *N*-demethylated metabolites vs. the C-8 oxidative metabolite ranged from 0.52 in the rat to 10 in the monkey. The ratio in humans was 2.78. *N*-3 demethylation was the major pathway in humans and rabbits (involving CYP 1A2), while *N*-7 demethylation predominated in monkeys (not mediated by CYP 1A1 or 1A2). Moreover, unlike that seen in the other species, rats and mice exhibit dose-dependent *in vivo* caffeine metabolism. In humans, mice, rabbits, and rats, the CYP1A2 isoform predominated over CYP1A1, although the ratios of these enzymes differed across these species (with negligible amounts of CYP1A1 detected in humans and mice). In monkeys, no CYP1A isoform was detected. These findings are consistent with the substantial discrepancy noted in the major P450 enzymes across the four major toxicological test species: dog, rat, rabbit, and mouse (106).

Soucek and Gut (107) have summarized the DNA sequence homologies between various rat and human P-450 isoforms. For numerous P450's, sequence homology of >75% was observed between rat and man. However, the potential for a difference in enzyme activity when a change in even one amino acid occurs should be considered when predicting the kinetic consequences of these similarities. The authors also noted upon a review of the literature that gene expression in rats is highly dependent upon such variables as gender (2A1, 2A2, 2C7, 2C11, 2C12, 2C13, 2D1, and 2A2), age (2A1, 2A2, 2B1, 2B2, 2C6, 2C7, 2C11, 2C12, 2C13, 2D, 2E1, and 3A2), strain (2B1, 2C13, and 2D1), circulating levels of growth hormone, and the physiological status of the animal (e.g., the effect of starvation, blood pressure, and diabetes). (As a note of caution, it should be recognized that this information is provided as a starting point for further consideration, but

that there currently is no universal agreement as to which specific isozyme is affected at any particular point in time).

Monkeys appear to express a higher proportion of reduction reactions associated with aldehyde oxidase as compared to that seen in other mammalian species. Aldehyde oxidase, an enzyme closely related to xanthine oxidase, is involved in the reduction of sulindac to sulindac sulfoxide and the reduction of imipramine *N*-oxide to the active parent drug, imipramine. In the presence of electron donors, it also mediates the reduction of sulfoxides, *N*-oxides, nitrosamines, azo dyes, oximes, epoxides, hydroxyamic acids, aromatic nitro compounds, and 1,2-benzisoxazole derivatives (108). In their study, Kitamura et al. observed that the aldehyde oxidase activity of cynomolgus monkeys was at least 3-fold greater than that of guinea pigs, rabbits and rats. This enzyme was absent in dogs. Accordingly, it was concluded that unlike that seen in other mammalian species, the aldehyde oxidase in monkeys functions as the primary reductase enzyme for many compounds, and that the reductase activity of the P450 system has a minor role in this species.

Despite the evolutionary proximity of humans and monkeys, large differences in Phase I and II enzymatic reactions exist (109). Using human and rhesus monkey liver microsomes, the P450 content of the monkey microsomes was approximately 3-fold greater than that seen with human samples. Six *in vitro* Phase I activities were markedly higher in the rhesus monkey as compared to humans. These included reactions involving erythromycin and benzphetamine *N*-demethylation (primarily CYP3A3 and 3A4), pentoxyresorufin *O*-dealkylation, ethoxyresorufin *O*-deethylation (CYP 1A1/1A2), ethoxycoumarin *O*-deethylation (CYP2E1), and chlorpromazine *S*-oxygenation. Although ethoxycoumarin *O*-deethylase activity was significantly higher in the rhesus monkey as compared to human microsomal samples (which would suggest differences in CYP2E1 activity), there was no difference in the 2E1-catalyzed *N*-nitrosodimethylamine *N*-demethylation. Coumarin 7-hydroxylase activity was the only Phase I reaction that was higher in humans as compared to monkeys (109) and is consistent with other reports of humans having higher coumarin 7-hydroxylase activity as compared to mice, rabbits, and guinea pigs (110). Rat liver microsomes do not appear to express the activity of this enzyme (111).

The studies by Stevens et al. (109) included an evaluation of the flavin-containing monooxygenases (FMO). In rhesus monkeys, significantly higher rates of cimetidine *S*-oxygenation and chlorpromazine *N*-oxygenation, suggested that *S*- and *N*-oxide formation via FMO constitutes a greater portion of drug oxidations in rhesus monkeys as compared to humans. For the Phase II metabolic reactions, uridine diphosphate glucuronosyltransferase (UDPGT) activity was almost seven times higher in rhesus monkey microsomes as compared to human. Sulfation reactions showed no differences

with regard to 17α -EE sulfotransferase, but cytosolic acetaminophen sulfotransferase was 4-fold higher in the rhesus monkey. Glutathione (GSH) conjugation (which is important in the detoxification of electrophilic alkylating agents) also tended to be higher in monkeys than humans. In contrast, hepatic *S*-methyltransferase activity (which is important in the metabolism of thiopurines) tends to be significantly higher in humans as compared to the rhesus monkey.

Subsequent studies from that laboratory were expanded to include dog and cynomolgus monkeys (112). Interspecies differences were again observed (Fig. 1). The investigators note that even when a particular pathway is present in multiple animal species, interspecies differences in K_m and V_{max} need to be considered (Fig. 2). Although substrates used were known markers for the human isoforms, these results underscore the vastly different metabolic profiles that should be anticipated across species, and the potential for these differences to result in species-specific drug effects.

Variations in enzyme kinetics (K_m and V_{max}) can result in marked interspecies differences in drug clearance and associated drug–drug interac-

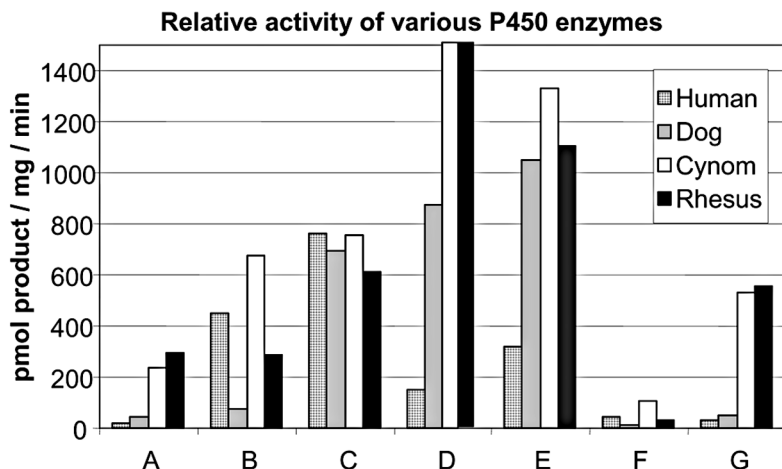


Figure 1 Interspecies differences in the relative activity of the various P450 enzymes. Key: A = ethoxy-resorufin *O*-deethylase; B = coumarin 7-hydroxylase; C = NDMA *N*-demethylase; D = erythromycin *N*-demethylase; E = midazolam 1'-hydroxylase (with 10 μ M midazolam) F = *S*-mephenytoin 4'-hydroxylase (with 25 μ M *S*-mephenytoin); G = bufuralol 1'-hydroxylase (with 10 μ M bufuralol). (Note that values for D in the two monkey species extend beyond the graph and have been truncated for the sake of illustration. Actual mean values in cynomolgus and rhesus monkeys are 2949 and 1997 et al. pmol/product/mg/min, respectively). Adapted from Sharer et al. (112)

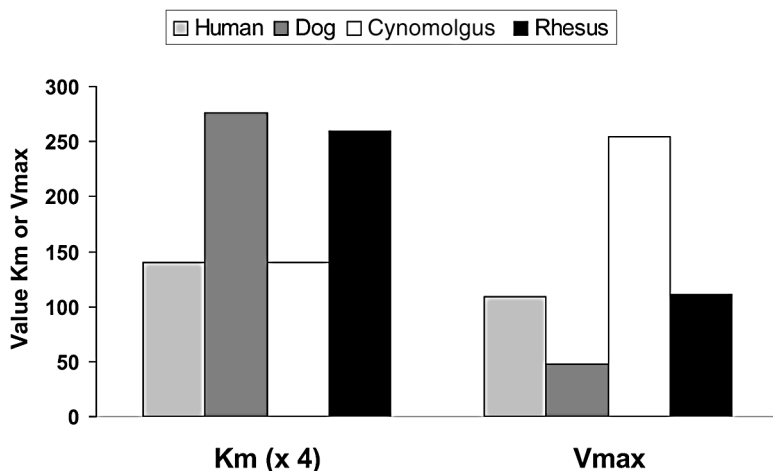


Figure 2 Interspecies difference in K_m and V_{max} for *S*-mephenytoin 4'-hydroxylase (K_m = micromoles, V_{max} = pmol product/mg/min). Note that for graphic purposes, all K_m values were multiplied by a factor of 4. Adapted from Sharer et al. (112).

tions. For example, in the case of 5,6-dimethylxanthene-4-acetic acid (DMXAA), mice and rats form the same metabolites as humans and, from a qualitative perspective, would be considered appropriate preclinical species for this compound (97). However, based upon the results of an in vitro microsomal preparation, no one species could consistently predict the extent to which specific inhibitors reduced the rate of glucuronidation and hydroxylation of DMXAA in humans (Fig. 3). Moreover, since these reactions exhibited Michaelis–Menton kinetics, the relative interspecies difference varied as a function of inhibitor concentration.

Glucuronidation is the most common conjugation pathway in mammals (113). These reactions are classified on the basis of the atom to which the glucuronic acid moiety is transferred: *O*–, *S*–, *N*–, and *C*–. The enzyme involved is UDP-glucuronosyltransferase. While *N*-glucuronidation generally is involved in detoxification reactions, in some cases (e.g., arylamines), this metabolite is believed to mediate the toxic effect of the parent compound (93).

Substrates for *N*-glucuronidation fall into one of two categories: compounds that form non-quaternary *N*-conjugates (e.g., sulfonamides, arylamines and alicyclic, cyclic and heterocyclic amines) and those that form the quaternary conjugates (e.g., tertiary amines such as the tricyclic antidepressants and antihistamine drugs). For the non-quaternary conjugation reactions, there is no laboratory animal species that exhibits a deficiency

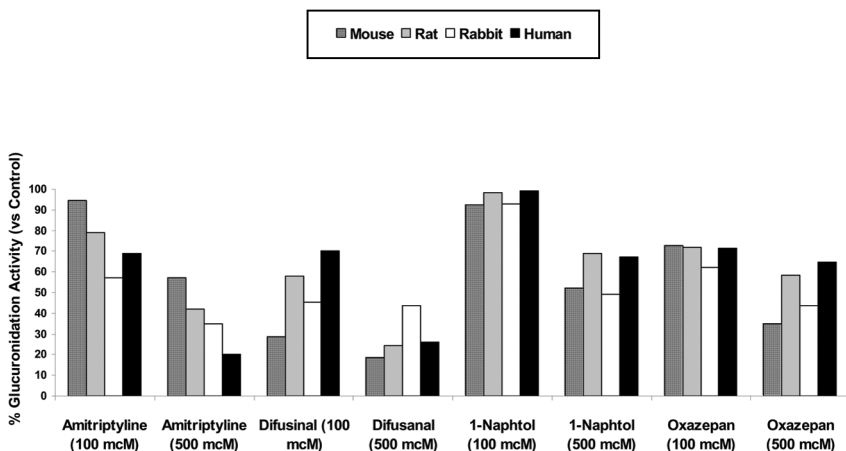


Figure 3 Interspecies differences in drug–drug interactions as demonstrated by the inhibitory effects of various compounds on the glucuronidation of DMXAA. Data are expressed as the mean percentage of glucuronidation activity remaining in Brij 58-activated pooled microsomes from humans ($n=3$), rat ($n=6$), mouse ($n=15$) and rabbits ($n=2$) at 100 or 500 μM inhibitor concentration. Adapted from Zhou et al. (97).

when all of the substrates are considered. However, the ability of a species to form these conjugates is compound dependent, and the rabbit and guinea pig appear to exhibit the highest capacity for this reaction among the various preclinical species including the rat, mouse, dog and non-human primate. For the tertiary amines, *N*-glucuronidation is commonly observed in non-human primates and man. *N*-glucuronides can be excreted in both the urine and bile of animal species (93).

When examining substrates possessing sites for both *O*- and *N*-glucuronidation, only the *N*-glucuronide metabolite was formed in human and canine microsomes, while both *O*- and *N*-glucuronides were formed in microsomes of monkeys and rats. This suggests the involvement of different UDP-glucuronosyltransferase isoenzymes in these reactions, and accordingly, differences in these isoenzymes across animal species (114).

There is much interest in the use of in vitro metabolism data to support the selection of animal species used in preclinical tests of a particular drug candidate (92). One of the problems when using in vitro test methods is the potential for interspecies difference in the conditions that optimize in vitro drug metabolism. For example, the optimal pH for *N*-glucuronidation reactions is 5.0 for the liver microsomes of monkeys and humans and 6.2 for the microsomes from dogs and rats. Another potential problem is that the microsomal preparation may not adequately reflect the in vivo substrate competition for a single metabolic pathway (114).

5.3. Age-Dependent Changes that can Affect Drug Pharmacokinetics

Unlike the other sections in this chapter, this particular section focuses largely on information obtained in humans. The reason for this diversion is due both to the scarcity of information on the impact of maturation on the pharmacokinetics in preclinical animal species, and because of the importance in recognizing the many ways in which adult animal or human data will fail to reflect the markedly different physiology and metabolism of juveniles.

On December 13, 1994, the FDA published a final rule encouraging manufacturers to provide information in product labeling that support the safe and effective drug use in the pediatric population (59 FR 64240). According to the 1994 Proposed Pediatric Rule (59 FR 64240), pediatric populations are defined as follows:

- Neonate: birth to 1 month
- Infant: 1 month to 2 years
- Children: 2 years to 12 years
- Adolescent: 12 years to <16 years
- Adult: ≥ 16 years.

To date, the majority of preclinical safety information associated with pediatric indications has been based upon studies conducted in healthy animals. However, as our knowledge base evolves, it is becoming increasingly evident that such studies may not be appropriate for identifying the potential drug toxicities associated with use in pediatric populations. Certain adverse effects may be relatively rare events that may be difficult to detect in clinical trials or during routine postmarketing surveillance. In other cases, the expression of a pharmacological insult may not be apparent until several years after drug use. For this reason, CDER recommends the use of juvenile animals for preclinical toxicity assessments of drugs intended for use in pediatric populations (115). A comparison of human to animal developmental stages across various organ systems and animal species are provided in the CDER draft guidance titled "Nonclinical Safety Evaluation of Pediatric Drug Products" (115).

At least in part, age-related differences in drug response may be attributable to the influence of maturation on drug absorption, distribution, and metabolism. In his survey of age-related differences in the pharmacokinetics of a wide range of compounds, Renwick (116) observed the following general trends:

- Children tend to eliminate drugs more rapidly than do adults.
- For renally cleared compounds, elimination is markedly slower in neonates as compared to other age groups.
- There are some drugs that show age-related shifts in body weight adjusted clearance (such as amrinone, meropenem, midazolam, and

cefotaxime). However, other drugs reach adult-like clearance values after the first few months of life (such as zidovudine, amikacin, and ketamine).

Clark University, in cooperation with the Connecticut Department of Public Health, created an extensive pediatric database containing published information across a wide range of pharmaceutical substances (117). The database categorizes information in accordance with clearance pathways and specific age groups. Information can be downloaded into Excel spreadsheets for further examination. Along with allowing for the determination of specific trends within an age group, this database was constructed to facilitate an age-related comparison of the magnitude of inter-subject variability associated with drug pharmacokinetics. This is particularly important given the variability in growth and maturation rates across individuals. For individuals interested in surveying an extensive comparative child/adult pharmacokinetic database, this information can be downloaded from [http://www2.clarku.edu/faculty/dhattis/#Child/Adult Database](http://www2.clarku.edu/faculty/dhattis/#Child/Adult%20Database).

On the basis of information contained within this database, and consistent with the findings of Renwick (116), Ginsberg et al. (118) draws the following conclusions:

- Premature and full-term neonates tend to have a 3–9-fold longer terminal elimination half-life as compared to that of adults. This difference generally disappears by 2–6 months of age.
- Across a variety of compounds (reflecting different degrees of extravascular drug distribution and clearance pathways), there is a trend towards a shorter terminal elimination half-life within the 6-month to 2-year age group as compared to adults. This difference seems to be related to enhanced drug clearance (weight corrected) in infants.
- Across a range of P450 substrates, the terminal elimination half-life of neonates and infants up to 2 months of age tends to be significantly longer than that associated with adults. Conversely, for numerous compounds, the elimination half-life tends to be significantly shorter than in adults within the 6-month to 2-year age bracket. Since the latter age group tends to have a significantly larger (not smaller) volume of distribution, it would appear that this difference in half-life reflects a higher level of Phase I drug metabolism. However, it was noted that despite this general trend, the magnitude of these differences is highly compound specific.

The overall activity of the P450 system tends to be 50% higher in adults as compared to the neonate. In general, enzyme activity reaches levels equal to or greater than that in adults within 6–12 months of age, with the total hepatic P450 content approaching adult levels during the first 10 years of life.

In children ages 6 months to 12 years, the activity of certain enzymes may be even higher than that seen with adults. This is believed to be linked to their inherently higher metabolic rate as compared to that of adults (119).

As a note of caution, it should be recognized that there are substantial differences in the age-related change in gene expression among the various enzyme systems. Moreover, there are minimal amounts of information regarding the factors involved in the activation of many of these systems. Accordingly, it is important to consider the differences in the rate of maturation for each of the various isoenzymes. For example, CYP3A7 is responsible for up to 85% of the total P450 activity in the fetal liver, but declines to adult levels by 12 months of age. Conversely, CYP3A4, which is not present in the fetal liver, becomes the major P450 isozyme shortly after birth and remains such for the remaining lifetime (120,121).

The impact of the maturation processes on the activity of Phase II metabolic pathways has resulted in dissimilarities in the handling of drugs such as acetaminophen. Approximately 50% of the administered acetaminophen dose is eliminated as the sulfate conjugate in children up to 12 years of age, while 50% of the administered dose is eliminated as the glucuronide conjugate in adults (122). This underscores the importance of considering the specific isoenzymes when predicting age-related changes in drug disposition. For example, there are 16 different UDP-glucuronosyltransferases, each with slightly different substrate affinities (123). The individual isoenzymes do not necessarily attain adult levels at same rate. Glucuronidation of simple substrates is higher at birth and subsequently decreases to adult levels by day 7. Conversely, the glucuronidation of bulkier substrates (such as chloramphenicol) is low at birth, and subsequently increases to adult levels by day 20 (124).

In humans, differences in body water, serum protein composition, and the affinity/capacity of hepatic biotransformation are observed between adults and pediatric patients (119,125). Many of these differences are particularly apparent when comparing adults vs. neonates. A summary of some of the differences influencing drug pharmacokinetics is provided in [Table 4](#) [based upon information contained in de Zwart, et al. (119), Clewell et al. (121), and Kearns and Reed (125) unless otherwise noted].

Developmental changes in the renal function of humans and rats appear to be similar. For example, the glomerular filtration rate in 10-day-old rats is 50% that of the adult. Filtration rate remains low for several weeks. By week seven of age, renal blood flow and glomerular filtration rate reach adult values (124). Relative kidney weight also changes dramatically with age (121). At birth, the ratio of kidney weight to body mass (1%) is twice that of the adult (0.5%). From birth through adolescence, kidney weight (expressed as a fraction of total body weight) declines. Change in kidney weight scales to the 3/4 power of body weight. Interestingly, this is

Table 4 Physiological Changes Associated with Maturation in Humans

Gastric volume (fasted)	2.5 mL for neonates, 8.8 mL for children and 50 mL for adults. Volume can increase approximately 50 fold after feeding.
Gastric acid secretion	Neutral pH at birth but falls to between 1.5 to 3.0 within hrs. Gastric acid secretion (corrected for body weight) approaches adult values by 3 months of age.
Gastric emptying	During the neonatal period, peristalsis is variable and unpredictable, with prolonged cycles relative to that of adult. Gastric emptying time approaches adult values within 6 to 8 months of age.
Interdigestive motor activity	Shorter in children than adults.
Exocrine pancreas	Low enzyme activity during neonatal period, but during infancy, secretion gradually approaches levels seen in adults.
Bile acid production	Less production in neonates than adults. However, infants are capable of efficiently absorbing fats within the first year of life
Total Body Water (TBW)	Highest at birth, decreases steadily through the first year, plateaus between years 1–10. Separation between genders begins during adolescence when female TBW decline faster than does that of males. Adult males and females have similar rates of decline, although TBW is consistently lower in females as compared to males.
Total Body Fat (TBF)	Adipose tissue of the neonate may contain as much as 57% water and 35% lipids, whereas in adults, adipose tissue contains 26.5% water and 71.7% lipids. Total body fat increases up to 9 months of age, remains relatively constant from infancy through childhood (about 25–30%), and then dips during adolescence (about

Serum albumin and total protein	a 15% decline in males, 5% decline in females). During adulthood, TBF increases in both males and females at the same overall rate (approaching 30% in geriatric males, 40% in geriatric females), but males consistently maintain a lower TBF as compared to females. Less than adult values during neonatal period and early infancy, but approaches adult values by approximately 1 yr of age. The serum protein of neonates differ from those of adults in several ways including: • The presence of fetal albumin (absent by 1 month of age). • Lower levels of plasma globulins (equivalent by early childhood). • Presence of unconjugated bilirubin (equivalent to adult by 1 month). • Free fatty acids are higher in neonates, but are at adult levels in 1 month • α1-acid glycoprotein are lower in neonates, but normal levels by 1 yr of age.
Blood pH Hepatic Phase I reactions Phase II reactions	Lower in neonates than adults, 7.26–7.29 versus 7.35–7.45 for neonates and adults, respectively. There tends to be lower alcohol dehydrogenase activity, carboxylesterase activity and P450 activity in children and neonates as compared to adults. Several not at adult level until 5 years of age. Children and neonates tend to show lower glutathione-S-transferase activity, glucuronyl transferase activity, but higher sulfotransferase activity. Neonates are typically slow acetylators. However, by 12 months of age, approximately 62% of individuals are fast acetylators. By 3–4 years of age, European and white and black children of the US show NAT2 phenotypic characteristics equivalent to that of adults.
Renal function	There tends to be lower glomerular filtration and tubular secretion in neonates, infants, and young children as compared to adults.

the scaling relationship that many argue is appropriate for converting body mass to surface area (see section on allometric scaling).

With regard to volume of distribution, the largest changes occur within the first 12 months of life. Infants and neonates tend to have ~ 1.3 to 2.8-fold larger distribution volumes (per unit body weight) as compared to adults. After 1–2 years of age, the volume of distribution of most compounds tends to be similar to that of adults (119,121). This trend is seen both with lipophilic and hydrophilic compounds, corresponding to the higher TBW and lower serum protein binding seen in the very young.

The small intestinal villi of neonates tends to be broad leaf-shaped projections, rather than the elongated projections observed in adults. The length and diameter of the small intestine also increase from birth through adulthood, with up to a 40-fold increase in absorptive surface area. For the most part, maturation of the gastrointestinal tract occurs within 6 months after birth, after which most of the absorption processes are similar to that of the adult (119).

There tends to be a prolonged residence of a compound in the stomach of infants and neonates. From age 0 to 3 months of age, there also tends to be a higher gastric pH, and the nearly continuous presence of milk can either increase or decrease the bioavailability of compounds normally absorbed by the stomach. Despite the slower rate of gastric emptying observed in neonate, there is generally little difference in the extent of absorption for most compounds (exceptions to this are described in the following paragraph). There also appears to be slower intestinal motility of young infants and neonates as compared to adults. For this reason, there is generally a slower oral absorption of compounds in neonates and young infants as compared to children and adults (119).

Despite age-related differences in small intestinal surface area, there are occasions when the extent of absorption of a substance in children exceeds that observed in adults. This is particularly true for compounds that are actively transported, such as calcium and iron (119). Differences in oral absorption of lead are also known to occur, with 4–5 times higher bioavailability seen in neonates than adults, and 3–4-fold higher bioavailability in children aged 2–6 as compared to adults. The mechanism for this difference is unknown (121). While it may, in part, reflect active transport via enterocyte receptors involved in the absorption of iron and calcium, there has been some suggestion of enhanced pinocytotic activity in early stages of development (126).

In neonates, the absorption of highly lipophilic molecules (including lipid-soluble vitamins) tends to be substantially lower than that observed in adults. This is attributable to a deficiency in the secretion both of pancreatic lipases and of the bile salts. For neonates and infants fed breast milk, the necessary enzymes for lipid digestion are derived from both

lingual lipases and from those lipases contained within the breast milk itself (119).

Interspecies comparisons between humans and preclinical species are generally based upon *in vitro* metabolism data (124). The observed inclination is for the relationship between postnatal age and drug metabolism to exhibit trends similar to that observed in humans, such as the generally lower glucuronidation occurring in the very young. A summary of changes in P450 isoenzymes in humans and animals can be found at <http://www.icgeb.trieste.it/~p450srv/P450ageing.html>

Even when we are able to accurately predict differences in drug pharmacokinetics that may exist between a pediatric vs. adult population, unexpected toxicities have been known to occur. There may be critical windows of organ sensitivity that would not be evident in toxicity testing conducted in adult animals, and the dynamic processes of growth and development may result in the manifestation of toxicities that are not evident until a later stage of growth and maturation (119). These challenges underscore the importance of conducting toxicity testing in developmentally age-matched animals.

5.4. Protein Binding Characteristics

Drugs can potentially bind to a variety of serum proteins including albumin, α_1 -acid glycoproteins, lipoproteins, sex hormone binding proteins, and immunoglobulins. They may also enter and bind to erythrocytes. Basic (cationic) drugs such as many β -adrenergic antagonists and macrolide antimicrobial agents are bound primarily to the α_1 -acid glycoproteins. Conversely, acidic (anionic) drugs such as furosemide, β -lactams, salicylate, and phenylbutazone tend to bind to serum albumin (127).

Since it is predominantly the free drug concentrations that are responsible for the physiologic effects of a compound, failure to identify interspecies differences in total vs. free drug concentrations may bias the interpretation of interspecies deviations in exposure/response relationships. For highly bound drugs, small differences in percent protein binding (e.g., 95% vs. 99%) can result in very large discrepancies in free fraction (e.g., a 5-fold greater free fraction found with 95% binding as compared to 99% protein binding). Moreover, free drug fraction can affect drug clearance. While variations in protein binding are expected to have minimal effect on the clearance of drugs associated with a high extraction ratio, the clearance of low extraction ratio drugs are likely to be diminished in the presence of high-level plasma protein binding (127–129).

Examples of marked interspecies differences in free fraction are provided in Table 5 [based upon Cayen (95) Mahmood (130)]. As evidenced from the table, protein binding tends to be highest in humans and lowest in mice.

Table 5 Free Fraction of Drugs Across Blood of Various Species

Drug	Mouse	Rat	Dog	Monkey	Human
Cefpiramide	0.56	0.54	0.70	0.068	0.037
Cefoperazone	0.854	0.744	0.744	0.161	0.176
Cefmetazole	0.65	0.56	0.75	0.19	0.15
Diazepam		0.137	0.04		0.032
Quinidine	0.363	0.324			0.13
Valproic acid	0.881	0.366	0.215		0.052
Meloxicam	0.04	0.003			0.004
CIPB	0.65	0.25	0.15	0.05	0.03
Etodolac	0.052	0.007	0.017	0.012	0.008
Tolrestat	0.04	0.017	0.02	0.014	0.007
Pelrinone	0.78	0.28	0.20	0.21	0.11
Benoxaprofen	0.011	0.007	0.008	0.004	0.002

Marked interspecies differences in plasma protein composition have been observed (131). Using a combination of the Biuret method for total protein measurement, the bromocresol sulfophthalein technique for quantifying blood levels of albumin, and electrophoresis to obtain estimates of the relative content of various plasma proteins, comparisons have been made across eight mammalian species. These differences are summarized in Table 6.

Again, there is a note of caution that the relative amounts of the various plasma proteins are not necessarily predictive of the relative extent to which a drug will bind to the plasma proteins of a particular animal species. However, knowing the interspecies difference in the extent of plasma protein binding for a particular compound may help explain some of the apparent

Table 6 Range of Plasma Protein Values of Eight Mammalian Species^a (131). Reprinted with permission

Parameter	Units	Mouse	Rat	Rabbit	Dog	Sheep	Man	Cow	Horse
Albumin	g/dL	3.5	2.1	3.9–4.3	3.2–3.8	3.1–3.5	4.6–4.9	2.2–2.4	2.7–3.1
Total protein	g/dL	6.0	6.5	6.7–7.4	5.6–5.9	6.7–7.6	7.2–8.4	5.8–6.8	6.0–7.8
$\alpha 1$	%		7	4–10	2–4	4–5	1–2		2–3
$\alpha 2$	%		5	3–15	8–10	9–11	6–8		7–10
β	%	12	12	14–16	20–23	21–26	10–11	30–37	13–18
γ	%	7	7	15–21	4–7	15–18	15–22	13–24	24–29
Albumin	%	59	68	55–60	58–64	46–49	59–65	32–41	40–50

^aMouse and rat blood represent pooled samples.
n = 4 per species. Each animal's samples were run in triplicate.

differences in drug pharmacokinetics across animal species. For example, the interspecies differences in diazepam total plasma clearance and terminal elimination rate constant were highly correlated with free fraction (Fig. 4A and B). Within human subjects, a similar correlation between plasma clearance and free fraction was also noted. No correlation between free fraction and the volume of distribution was observed for either humans or animals (Fig. 4C). When converting plasma clearance to total body clearance and subsequently to extraction ratio (total body clearance/hepatic blood flow), man was found to have a low extraction ratio for this compound while dogs, rabbits, and rats were found to have high extraction ratios. In some cases, values of E exceeded 1.0, indicating the presence of extrahepatic elimination processes (132). A high E value also suggests that hepatic clearance will be affected by variables that can alter hepatic blood flow (e.g., food), but not by changes in plasma protein binding. In man, the value of $E < 0.2$ is consistent with an elimination process that is highly dependent upon free fraction, not hepatic blood flow. Accordingly, a linear correlation was observed between clearance and free fraction across the human subjects.

Interspecies differences in protein binding may not reflect differences in the drug–protein interaction but may rather be attributable to the presence of other substances that compete for the protein binding site. Alendronate (an inhibitor of osteoclast-mediated bone reabsorption) binds to both plasma proteins and to bone. Irreversible binding to bone constitutes the primary mechanism of drug elimination (133). Relatively low plasma protein binding was seen in dogs, but high protein binding was observed in rats. This interspecies difference was, at least in part, attributable to the apparent presence of displacers(s) in dog but not rat plasma (based upon *in vitro* experiments). The addition of calcium to the dog plasma sample diminished the effect of the displacer(s).

Alternatively, interspecies differences in protein binding may reflect differences in protein binding affinity. The affinity of fatty acid-acylated insulin for serum albumin of humans, pigs, and rabbits (expressed relative to the binding affinity to human albumin) varied from 1:1.5:35, respectively. As a result of the much higher binding affinity of this compound in rabbits, the fatty acid-acylated insulin exhibited a diminished but prolonged effect in rabbits as compared to pigs (134).

Interspecies differences in Michaelis–Menton binding characteristics have also been observed. For example, the basic compound, propafenone, was found to have at least a 2-fold higher free fraction in rabbits as compared to that of other species. There were also marked differences in the dose-dependency of protein binding across species. As the concentration of propafenone increased from 250 to 2000 ng/mL (*in vitro* test procedure), non-linear protein binding was observed in horses (2-fold change), mouse (3-fold change), man (2-fold change), and sheep (5-fold change). However,

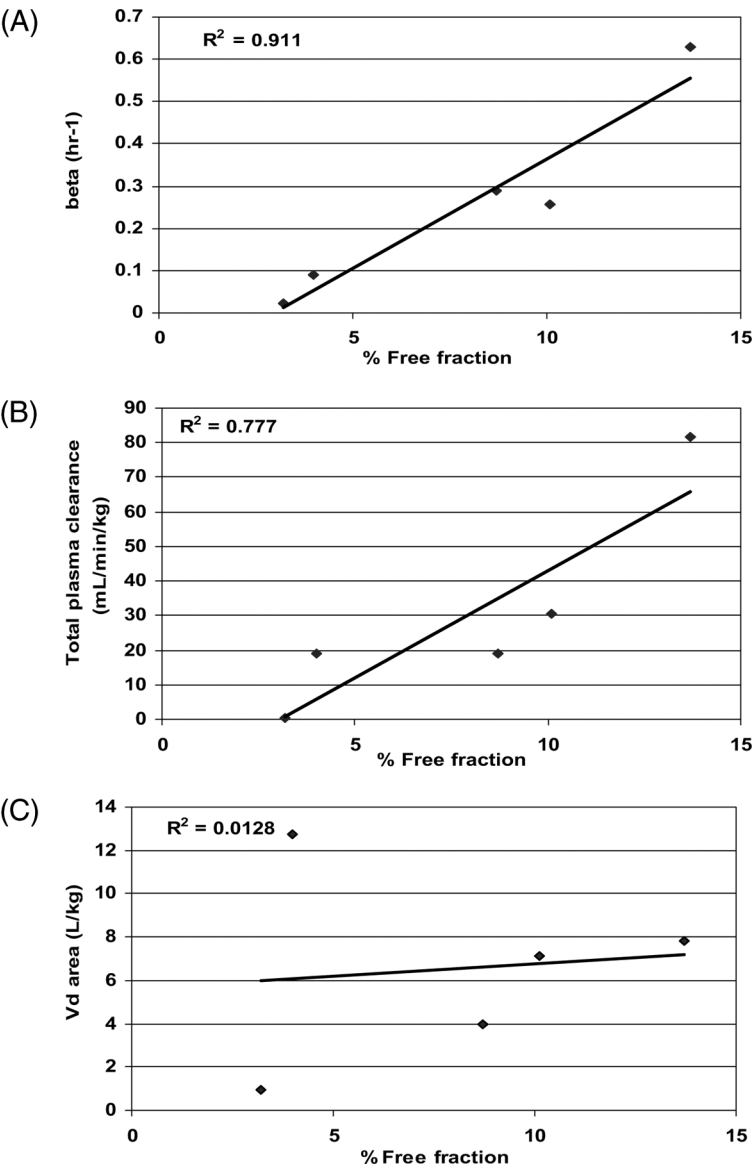


Figure 4 Interspecies relationship among pharmacokinetics parameters. (A) Free fraction vs. terminal elimination rate constant. (B) Free fraction and total plasma clearance (C) Free fraction and volume of distribution. Plots are based upon data reported by Klotz et al. (132).

dose-independent protein binding was observed in rats, rabbits, dogs, and cattle (131).

5.5. Biliary Excretion

Interspecies differences in biliary excretion can lead to pronounced differences in drug exposure, particularly when the drug undergoes enterohepatic recirculation. In general, the extent of biliary excretion tends to be much higher in dogs and rats as compared to pigs, monkeys, and humans. The mouse falls somewhere in between these two groups (95).

The marked interspecies differences in mean bile flow and composition can also affect drug solubilization and therefore drug absorption (135,136). The differences in bile flow across target animal species are summarized in Table 7. Although rats and horses have no gall bladders, both species synthesize bile salts and bile entry to the intestine occurs in a more or less continuous manner.

Interspecies differences in drug metabolism may also influence the extent of enterohepatic recirculation for a particular compound. In the case of oxaprozin, an anti-inflammatory agent, it was found to undergo both oxidative metabolism as well as glucuronidation. The glucuronide is not formed in rats, is excreted primarily in the urine of humans, is found in both the urine and bile of the rhesus monkey and is eliminated almost exclusively in the bile of dogs. Upon elimination in the bile, oxaprozin glucuronide is deconjugated in the small intestine by intestinal glucuronidases. The parent drug is thereby regenerated and available to be reabsorbed (102).

Table 7 Mean Bile Flow (29). Reprinted with Permission

Species	Bile flow ($\mu\text{L}/\text{min}/\text{kg BW}$)
Cat	11
Chicken	20
Dog	4–10
Guinea pig	200
Hamster	50
Human	1.5–15
Monkey	10
Mouse	78
Pig	9
Pony	19
Rabbit	90
Rat	30–150
Sheep	9.4

Thus, human intestinal exposure to this drug would be underestimated on the basis of rat data but overestimated on the basis of dog data.

The extent of biliary excretion, if followed by enterohepatic circulation, can be an important factor contributing to the risk of drug toxicity. This point is clearly demonstrated with indomethacin where there is a distinct relationship between enterohepatic recycling, intestinal drug exposure, and toxic dose. When administered indomethacin, marked interspecies differences in cumulative intestinal exposure were observed, where dog > rat > rhesus monkey > guinea pig > rabbit > man. The corresponding toxic dose in these species was related to the magnitude of their intestinal indomethacin exposure. Therefore, while the toxic dose was only 0.5 mg/kg/day in dogs, it was as high as 20 mg/kg/day in rabbits (137). Similarly, unusually high bile/plasma concentration ratios of the sulfasalazine analogue, susalimod, were observed in dogs (ratio = 3400) as compared to monkey (ratio = 300) and rat (ratio = 50). This difference in bile concentrations correlated with the long-term use hepatobiliary toxicity observed in dogs but not in the other two species (138).

Since presence or absence of a gall bladder also impacts the characteristics of bile release into the intestine, we anticipate that the presence of a gall bladder and the pattern of bile release in the intestine will influence the rate and extent of biliary drug recycling. In species with gall bladders, the discharge of bile into the duodenum occurs during Phase II of the migrating motor complex (139). The latter is a myoelectric cycle, originating in the stomach and propagating throughout the intestine (140). Since rats lack a gall bladder, these fluctuations are not observed. Rather, bile flow appears to follow a circadian pattern, with secondary (superimposed) variation occurring as a result of food intake (141).

Efficient biliary excretion of a compound is both a function of the molecular weight, chemical nature, and target animal species. The molecular weight threshold for the biliary excretion of acidic compounds is approximately 300–350 in dogs and rats but greater than 500 in humans. Similar molecular weight considerations apply to most neutral compounds (102).

6. ALLOMETRY

Allometry serves as a black-box approach for interspecies scaling of drug concentrations within some biological matrix (generally blood). While there are numerous examples of its successful application (142,143), there are also examples of where allometry fails to accurately predict drug pharmacokinetics across species.

The variable generally considered to be the most highly predictive factor for interspecies scaling is total body surface area. This is because pharmacokinetic elimination processes are affected by the size and function of the eliminating organ, which in turn, reflects the organisms' metabolic

demands. In turn, metabolic rate appears to be related to total body surface (44,144). Therefore, it is not surprising that many of the pharmacokinetic elimination processes scale in accordance with total body surface area.

This leads to the issue of how to obtain an estimate of body surface area across the various animal species. To address this point, West et al. (145) suggest that the biological commonality supporting interspecies scaling is founded upon certain general principles:

- Living things are sustained by the transport of materials.
- Transport occurs through linear networks that branch to supply the various parts of the organism.
- This network can be characterized as a space-filled fractal-like branching system.
- The final branch of this system (e.g., the capillary) is a size-invariant unit.
- The energy required to distribute resources are minimized in all living creatures.

These authors suggest that an outcome of these principles is an inherent scaling relationship between mass and surface area.

From a naïve approach, the belief is that the relationship between mass and surface area is related to the need to release body heat through the surface area of the organism. In other words, the fundamental relationship is that of metabolic rate, body mass, and surface area. By convention, surface area is assumed to scale in accordance with $V^{2/3}$, where V = volume of the organism. In turn, V is considered to be proportional to mass, under the conditions of a constant body density (146). Subsequent reports, however, have suggested that the power relationship between mass and metabolic rate is that of 0.72–0.73 rather than of 0.67. West et al. (145,147) provide several theoretical and mathematical arguments supporting the use of a 3/4 rather than 2/3 power for converting mass to surface area.

In an attempt to reconcile this debate, Dodds et al. (147) examined the theoretical attempts to connect metabolic rate to mass, as described by the equation:

$$B = cM^\alpha$$

where B is the basal metabolic rate, M the mass of the organism, α the allometric exponent and c a constant.

They examined several mathematical models to cover such theoretical approaches as dimensional analysis, four-dimensional biology, and nutrient supply networks. They concluded that none of these theories convincingly support a 3/4 rather than 2/3 scaling relationship. [Interestingly, they also examined the work of West et al. (145), and raised concerns regarding the assumptions and mathematical accuracy of West's arguments supporting the conclusion that surface area scales to mass by the 3/4 power.]

Dodds et al. (146) also examined empirical data for metabolic rates for homeotherms. They observed that based upon the actual metabolic data obtained from almost 800 species (including birds and mammals), they could not find statistical support for rejecting $\alpha = 2/3$. However, they also observed an apparent shift in α as body mass increases. Basically, below 10 kg, the allometric exponent of $\alpha = 2/3$ appears to fit well. As body mass increases above 10 kg, there is a greater-than-predicted increase in metabolic rate, and α appears to scale better to a factor of $3/4$ rather than $2/3$. They hypothesize that this shift may reflect a change in body shape with increasing size, and therefore a change in the surface area to mass relationship. In this regard, they note that the relationship between mammalian head-and-body length and mass is better fit by two rather than one scaling law, and suggest that a higher metabolic rate might provide an evolutionary advantage to support larger brain sizes. (It is interesting to contemplate the relationship between these suggestions and the other potential use of additional normalization factors, such as brain weight, as discussed later in this section.) They also note that α values shift across species of birds with different normal core temperatures (differing by $1\text{--}2^\circ\text{C}$) when metabolic rates are grouped to different seasonal measurements. On the foundation of these evaluations, they concluded that while a single allometric relationship may be useful for obtaining rough estimates of interspecies predictions, the assumption of a single allometric relationship across a wide range of weights may not be justifiable.

Similar to the equation relating basal metabolic rate to mass, the general form of the allometric equation used in scaling pharmacokinetic parameters across animals is as follows (148):

$$Y = aBW^b$$

where Y is the parameter of interest, BW the body weight, a the allometric coefficient (the value of the physiological variable (y) at 1 unit of body weight), and b the allometric exponent that defines the proportionality between W body weight and Y .

When $b = 1$, there is a direct correlation between body weight and the parameter, Y . When the constant equals 0.67 or 0.75, Y is said to scale in accordance with body surface area (149).

Conversions of body weight (kg) to body surface area (m^2) are provided in Table 8 [based upon Morris (20) and CDER guidance First time dose in man, 2002 (150)].

To explore the impact of data variability on the ability to distinguish between $b = 2/3$ or $3/4$, Hu and Hayton (143) examined the allometric relationship for 115 compounds. They found that 91 of the surveyed drugs exhibited a statistically significant allometric relationship. Estimated values of b ranged from 0.29 to 1.1. For drugs whose elimination included metabolism, the estimated values of b did not differ significantly from 0.75. Only in

Table 8 Conversion of Body Weight to Surface Area Across Species and Age Groups

Species	Body weight (kg)	Surface area (m ²)
Human, adult	60	1.6
Human, child	20	0.8
Mouse	0.02	0.007
Rat	0.15	0.025
Cat	3	0.24
Dog	16	0.65
Sheep/goat	50	1.1
Pig	75	1.5
Non-Human Primates		
Marmoset	350	0.06
Squirrel monkey	600	0.09
Baboon	12	0.06

the case of drugs that are cleared solely by renal elimination were the allometric exponents significantly lower than 0.75 (mean = 0.65, 95% confidence interval = 0.62–0.69). Given the shape of the distribution of these estimates, the authors suggest that for all drugs except those cleared solely by renal elimination, reported differences in the estimates of the allometric exponents are more a function of population variability and experimental noise than of real differences in scaling factors.

These authors further examined the issue of using $b = 0.67$ vs. $b = 0.75$ through the Monte Carlo simulation of 10 experimental scenarios. Each scenario differed with respect to the selection of sampling times, number of animal species, and coefficients of variation. Under various simulated experimental conditions, they examined the impact of study design and random error on the estimated allometric exponent. They noted that the resulting estimates of b followed a normal distribution similar to that observed with 115 actual datasets (discussed above). They further noted that with a 30% CV, it was impossible to determine whether the true value of b was 0.67 or 0.75 (simulations were based upon an assumed value of $b = 0.75$). Accordingly, they conclude that for most compounds, it will not be feasible to assume that one can establish a conclusive relationship based upon conventional experimental data and study designs. Using Monte Carlo methods, similar conclusions were reached by Watanabe et al. (151).

For any given parameter, there can be several versions of the allometric equation that is said to best fit the specified parameter. For example, two versions of the equation for estimating cardiac output (expressed in mL/min/kg) are $166 \times BW^{0.79}$ (142) and $15 \times BW^{0.74}$ (152). Reasons for these differences can include factors such as variability within each species, breed of animal selected, number of animals per species, and sampling times.

Species-specific idiosyncrasies in absorption, distribution, and metabolism often confound the use of interspecies extrapolation to predict appropriate dosages (amount and frequency) for use in humans or other animals. Factors such as interspecies differences in protein binding and metabolic pathway can result in failed attempts to accurately predict an appropriate dose from animals to humans (153). Therefore, allometric scaling tends to work best for those compounds that are eliminated primarily by physical transport processes such as biliary or renal excretion (154). Accordingly, allometric scaling tends to fail for those compounds that present with the following characteristics (20,154):

- Low extraction ratio ($E < 0.2$), where hepatic clearance is much less than hepatic blood flow.
- The presence of interspecies differences in drug metabolism.
- Non-linear pharmacokinetics.
- High protein binding (plasma and tissue).
- Renal tubular reabsorption. It should also be noted that the urine pH of herbivores tends to be alkaline while that of carnivores tends to be acidic, which may affect the renal clearance of certain compounds.

To determine whether or not drug physico-chemical properties influence interspecies pharmacokinetic relationships, the accuracy of extrapolating terminal elimination half-lives between rats and humans was considered with respect to drug lipophilicity (155). The question was not one of interspecies differences in membrane solubilization for specific compounds since a chemical's ability to be solubilized within tissues is assumed to be approximately constant across animal species (156). Rather, this question was raised in an attempt to address the substantially greater percentage of adipose tissue in humans (23% of total body weight) vs. rats (7% of total body weight). These investigators found that with the exception of a slightly higher prediction error when the human half-life was estimated for very highly lipophilic compounds (e.g., $\log P > 6.5$), only minor differences in prediction error occurred between models with or without the inclusion of $\log P$ as a factor in the regression equations.

The following physiological parameters tend to scale in accordance with body weight [i.e., the value of b tends towards unity (149,157)]:

- Organ volumes: blood volume: $b = 1.02$.
- Organ weight.
- Kidney weight: $b = 0.85$.
- Heart weight: $b = 0.98$.
- Liver weight: $b = 0.87$.
- Stomach and intestines weight: $b = 0.94$.
- Blood weight: $b = 0.99$.

In contrast, the following physiological parameters appear to be more closely linked to metabolic rate [i.e., approximately 0.75 (149,157)].

- Cardiac output: $b = 0.75$.
- Alveolar ventilation: $b = 0.75$.
- Creatinine clearance: $b = 0.69$.
- Inulin clearance: $b = 0.77$.
- PAH clearance: $b = 0.80$.
- Basal O₂ consumption: $b = 0.72$.
- O₂ consumption by liver slices: $b = 0.85$.

Despite differences in absolute amount of blood flow across species, as seen in Table 9, regional blood flow distribution, expressed as a mean percent of the cardiac output, was very similar across these four species.

If the allometric exponent for intrinsic clearance is the same as that for the blood flow of the eliminating organ, then the extraction ratio will be nearly identical across animal species (158). Accordingly, extraction ratio bears no relationship to body weight or body surface area. An example of this is propranolol, where the hepatic extraction ratio was estimated to exceed 90% in mice, dogs, and humans.

Marked prediction errors can occur if differences in drug metabolism are not adequately considered. A case in point is a drug that was shown to be toxic to the gonads of several animal species. It was originally considered safe for use in human use because, on the basis of surface area equivalents (allometry), it was determined that the animals would be exposed to seven times the level of drug expected for humans. However, when human

Table 9 Regional Blood Flow Distribution^a Expressed as percent Cardiac Output in Unanesthetized Animals (152). Reprinted with Permission

Tissue	Mouse	Rat	Dog	Human
Adipose		7.0		5.2
Adrenals		0.3	0.2	
Bone		12.2		4.2
Brain	3.3	2.0	2.0	11.4
Heart	6.6	5.1	4.6	4.0
Kidneys	9.1	14.1	17.3	17.5
Hepatic artery	2.0	2.1	4.6	
Hepatic vein	14.4	15.3	25.1	18.1
Lung	0.5	2.1	8.8	
Muscle	15.9	27.8	21.7	19.1
Skin	5.8	2.8	6.0	5.8

^aBased upon a compilation of data from studies employing a radiolabeled microsphere technique.

pharmacokinetic data became available, it was found that the exposure ratio was not a factor of seven but rather a factor of two (159). Unfortunately, knowledge of the P450 isoenzyme responsible for drug metabolism provides neither a guide as to the appropriate allometric exponent to use nor is it indicative of the overall ability to use allometric methods to predict human drug clearance (160).

In addition to use of total body surface area to predict allometric relationships for drug pharmacokinetics, differences between chronological vs. physiological time may also be considered when predicting interspecies differences in exposure response relationships. For example, cellular division rates in smaller animals are significantly faster than are those in large animals. This results in the former having a shorter latency for the proliferation of an immune response or the expression of an adverse cellular event. On the other hand, the larger animal species have a longer life span, resulting in a much longer time for the development of an adverse event (4).

Variables such as the duration of a single breath, heartbeat duration, longevity, pulse time, breathing rates, and blood flow are approximately constant across species when scaled to physiological time. In general, smaller, short-lived animal species clear drugs more rapidly (chronological time) than do larger, longer-lived animals. Since life duration tends to be related to body weight, the latter can be used to scale for differences in physiological time (142). The relationship between chronological time (t') vs. physiological time (t) has been expressed as (148,149):

$$t' = t/BW^{0.25}.$$

Dedrick et al. (161) were the first authors to suggest that interspecies scaling can be based on the concept of equivalent time. They proposed that drug elimination could be correlated between species if an intrinsic biological property such as creatinine clearance, heartbeat duration, longevity, breath rate and duration, or blood circulation velocity were used as an interspecies scaling factor. In other words, two apparently different rates of an event, when based upon chronological time, may in fact be comparable if adjusted to a species' physiological time.

This difference in physiological time can impart substantial influence on the toxic or therapeutic response to a drug. For example, the total blood volume in the mouse is 2 mL (162) and its cardiac output equals about 15–20 mL/min (163,164). Consequently, in mice, tissues are exposed to the entire blood volume several times each minute. In contrast, the cardiac output of the human is 1/20th of its total blood volume and it takes 5 min for the entire system to be exposed once to the total blood volume (4). Therefore, mice are likely to exhibit more rapid acute responses to toxic substances as compared to humans.

In general, the time for one complete systemic exposure to the entire blood volume of any species can be scaled as $Y = 0.35 \times BW^{0.21}$ (165). In this regard, Mordenti (165) notes that the blood volume turnover time for inulin can be scaled as $Y = 6.51 \times BW^{0.27}$.

Heartbeat time is said to equal $0.2961 \times B^{0.28}$ (where B is body mass in kilograms) (166). Hence, a 30-kg mouse has one heartbeat every 0.111 sec while a 70-kg human has one every 0.973 sec. Similarly, breaths per second (breath time) scales as $1.169 \times B^{0.28}$. These averages should be considered from the perspective of the wide range of factors that can influence these values such as exercise, gender differences, environmental temperature, posture (supine vs. standing), age, and the effects of a meal (152).

Boxenbaum (166) argues that when pharmacokinetic processes are similar across species, a pharmacokinetic parameter can be scaled to physiological time, thereby obtaining a time-invariant measure. This produces, in his terms, pharmacokinetic time. For example, the terminal elimination half-life for hexobarbital, based upon chronological time, can be described as:

$$T1/2 = 80 \times B^{0.348}.$$

The equation for normalizing for differences in physiological time [in this case, using a term coined gut-beat duration (G , min)] is:

$$G = 0.0475 \times B^{0.31}.$$

By dividing $T1/2/G$, one obtains a time-invariant terminal elimination half-life for hexobarbital ~ 1684 . The importance of estimating a time-invariant terminal elimination half-life is that that value can then be evaluated from the perspective of the rates associated with other physiological events occurring within that animal species.

Along similar lines, Boxenbaum and Ronfeld (167) introduced the concept of the kallynochron ($=t/W^{1-b}$) where one kallynochron defines the time within which species have cleared a specified volume of plasma per kg of body weight. Failure of the kallynochron to scale chlordiazepoxide pharmacokinetic data from dogs to humans lead these authors to further develop a term coined the apolysichron. The latter is defined as:

$$t/W^{b'-b}$$

where b' and b are the allometric exponents relating volume of distribution and clearance to body weight.

To illustrate how physiological time can scale the rate of a response, consider two pendulum clocks, each identical in form but one being 64 times

larger than the other (166). The duration of one cycle (swing) of the pendulum (T) can be defined as:

$$T = 2\pi(L/g)^{1/2},$$

where L is the pendulum length and g is the acceleration of the pendulum due to gravity.

The 64-fold increase in L produces only an 8-fold increase in T (i.e., $64^{1/2} = 8$), causing the larger clock to produce fewer ticks per minute. To compensate for this difference, the larger clock will need to have an 8-fold greater belt drive ratio to enable both the smaller and larger clocks to move through identical arcs per minute (representing chronological time). When this example is considered from a biological perspective, these differences in T (without adjustment from a “belt drive”) result in differences in life span and rates of physiological processes, as measured from the perspective of chronological time. However, with appropriate mathematical transformations, T can be expressed in a time invariant value.

When testing substances for potential carcinogenicity, the rate of carcinogenesis appears to relate to the species’ basal metabolic rate. For example, the onset of cancer often occurs within approximately one year in rodents but may take 10–20 years to be expressed in humans (17). On the other hand, upon relating this finding to physiological rather than chronological time, Dedrick and Morrison (168) observed that interspecies differences in the daily dose and AUC values associated with the development of cancer largely disappeared when adjustments were made for total lifetime exposure.

Ultimately, there are any numerous covariates that may be incorporated into an allometric equation to improve its predictive properties when scaling from animals to humans. To reduce some of the uncertainty associated with these allometric procedures, Mahmood and Balian (169) suggested a classification method for predicting the appropriate allometric exponent. Based upon additional scaling factors suggested by Boxenbaum and Dihea (44) and Boxenbaum (166), Mahmood and Balian considered the impact of including maximum life potential and brain weight on the allometric fit associated with interspecies datasets obtained from literature surveys. Based upon regression analysis conducted on 40 compounds, they developed the following conditions for determining the appropriate scaling method:

- If the exponent of the simple allometric equation lies between 0.55 and 0.70, a simple allometric equation can predict the clearance reasonably well. In this case, total body clearance (CL) would be estimated as follows:

$$CL = a(W)^b,$$

where W = body weight

- If the exponent of the simple allometric equation lies between 0.71 and 1.0, a prediction based upon the simple allometric equation will substantially overestimate the predicted clearance. In this situation, accounting for differences in maximum life span potential (MLP) appears to improve the fit. For this situation, *CL* would be estimated as follows:

$$CL = a(MLP \times CL)^b / MLP \text{ of humans}$$

where $MLP = 185.4 (BW)^{0.636} (W)^{-0.225}$, BW = brain weight, MLP of humans = 8.18×10^5 .

- If the exponent of the simple allometric equation is greater than 1.0, the product of *CL* and *BW* can be used to predict human *CL* with reasonable accuracy. For this situation, *CL* would be estimated as follows:

$$CL \times BW = aW^b.$$

- In cases where $b > 1.3$ or < 0.55 , neither of these three methods could adequately predict the *CL* of humans.

Under experimental conditions where drug pharmacokinetics can be examined across a wide spectrum of animal species, there is the luxury of being able to examine residual errors in order to determine the covariates that optimize the fit of the regression line. In so doing, the investigator can minimize the error in predicted vs. observed parameter values in humans [e.g., (170)]. However, what happens when one attempts to estimate a human equivalent dose (HED) on the basis of the NOAEL associated with the animal species of interest? In that situation, the fundamental objective is to ensure that the dose administered will result in negligible toxicity. This point brings us back to the debate described in the beginning of this section: is it more appropriate to scale to the power of 0.75 or 0.67? To that end, the use of an exponent of 0.75 rather than 0.67 will result in a far larger estimated starting dose in humans (e.g., a nearly 2-fold greater estimate when scaled on the basis of data derived from smaller rodent species, such as mice). Accordingly, the use of 0.75 could result in a higher and potentially more dangerous starting dose in humans. For this reason, the HED calculation is often based upon $b=0.67$ (150), thereby increasing the probability that the drug will be safe when administered for the first time in healthy human volunteers.

7. CONCLUDING THOUGHTS

While this chapter focused on animal models, comparative anatomy and physiology, and the extrapolation of preclinical data to humans, a far more complex question is whether or not preclinical data can also predict

toxicities that may be associated with a specific patient population. Numerous physiological changes can occur during disease conditions, and these changes can impact drug distribution, protein binding, clearance, drug metabolism, and tissue sensitivity. While we raise this question, we recognize that this point in and of itself can be the subject of an entire textbook. Nevertheless, it is a point worth considering as we use preclinical data to predict appropriate drug dosages in humans.

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Transgenic Animals for Preclinical Drug Development

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1. INTRODUCTION

The means to transform a physiologic pathway from one genotype to that of another is termed transgenic technology. In the realm of drug development, transgenic animals typically carry human physiologic pathways for efficacy and metabolism. This technology carries tremendous potential to elucidate potential human outcomes early in the lead optimization and preclinical development stages. Transgenic technology is a maturing area of science and, although it already provides considerable value, it is anticipated that its true value is yet to be realized.

Transgenic animals are produced in the laboratory by molecular manipulation of their genomes. The methods used to produce these animals have become routine practice in biomedical sciences over the past two decades. Although transgenic technology was first developed to address fundamental problems in biology, this technology has evolved rapidly over recent years and is now able to accommodate a wide variety of needs in different research areas, including basic biological sciences and application-oriented research such as drug development. Preclinical studies in drug development are intended to define the pharmacologic and toxicologic effects predictive of human responses. Animal models are essential tools for these phases of study; however, suitable animal models may not always be available.

Furthermore, as more biologically based therapeutic agents and delivery methods are invented, conventional pharmacology and toxicity tests may not always be appropriate to determine biological activity and to assess the safety of these agents. Transgenic technology facilitates the development of suitable animal models for testing conventional drug candidates and therapeutic agents that are derived from animal tissues (1). This chapter will provide an overview of different gene transfer methods to manipulate animal genomes and discuss their application in preclinical evaluation of new drugs, including assessment of the biological activities, metabolism, and toxicological effects of the drugs under development. Finally, a prospective view about transgenic applications in drug industry will be provided.

2. PRODUCTION OF TRANSGENIC ANIMALS

2.1. Principles

Two principal methods of changing animal genomes in the laboratory are random addition and targeted alteration of specific DNA sequences under investigation (Fig. 1). Random addition of genetic material into an animal genome is accomplished by introducing recombinant DNA directly into the pronuclei of oocytes that are then allowed to develop into mature animals (2). In technical terms, these animals are generally referred to as transgenic animals.

The second method is to introduce targeted alteration of genetic material at a specific genomic locus. This is accomplished by first changing a specific DNA segment in embryonic stem (ES) cells by a “gene-targeting” procedure (3,4), followed by the incorporation of the genetically altered ES cells into embryos which are then allowed to develop into mature animals. Animals derived from these embryos are generally called chimeric animals because they are composed of two genetically distinct cell populations (5). One cell population is derived from the recipient embryos and the second is derived from the genetically altered ES cells. By breeding the chimeric animals to a strain that is genetically distinct from the source of ES cells, animals with one set of chromosomes inherited from the ES can be selected among the offspring according to ES-specific genetic markers. Technically, these animals are referred to as gene-targeted animals because they inherit the targeted gene on one set of chromosomes that are derived from the ES cells. These gene-targeted animals, usually at a heterozygotic state, are subsequently bred within the same line to generate homozygote animals carrying the same targeted gene in both sets of chromosome. These animals are homozygous, gene-targeted animals.

To achieve a higher level of specificity in terms of where and when a specific gene-targeting event should occur, a more sophisticated approach combines the use of transgenic and gene-targeting procedures.

By incorporating a transgenic system that carries a regulating mechanism into the gene-targeted animals, it is then possible to conduct a regulated gene-targeting experiment (6,7).

For specific purposes, genetic alteration can also be manipulated at the level of somatic cells without passing through the germ cells. These are generally conducted by delivering recombinant DNA into specific target tissues or cell types without intervening with animal reproduction. This type of animal model is required to evaluate the pharmacology and toxicology associated with human gene therapy (8) and is specifically designed to meet guidelines for human gene therapy protocols that must avoid changes in germ cells and requires tissue or cell type specificity.

To produce transgenic animals (Fig. 1), two technical issues must be addressed: how the recombinant DNA will be designed and how the material will be introduced into the animal genome. The first issue concerns the purpose of a given experiment. This can be to investigate the function of a gene product, to define the regulatory elements for a particular gene, to evaluate the biological role of cells that express this gene, to address physiological problems as a result of expressing an abnormal gene, or simply to produce some useful gene products. The second issue concerns the route of recombinant DNA delivery into animal genomes that can be subsequently inherited in the offspring in a stable manner. The progress in molecular biology has made it possible to design recombinant DNA molecules for a variety of purposes. Therefore, the first technical issue has been addressed long before the maturation of the transgenic technology. However, delivery of genetic material into animal germ lines appears to be more of a technical challenge than a conceptual problem. Below, highlights in the development of this technology are briefly reviewed and technical information is summarized. Relevance to preclinical studies is also given.

2.2. Experimental Approaches

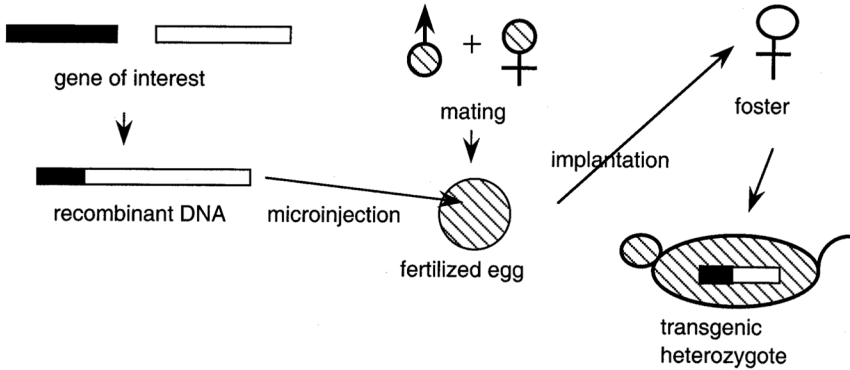
2.2.1. Design of Recombinant DNA Cassettes

The design of recombinant DNA depends upon the purpose of a specific experiment. In general, the purpose can be to ectopically express a gene product (wild type or mutated) for “gain or loss of function,” to examine the regulatory mechanisms of a particular gene, or simply to produce useful proteins for therapeutic applications (9,10). Modifications of these strategies have also been developed for specific purposes. For instance, cell ablation can be achieved by introducing a toxic gene into specific cell types (11,12). This is particularly useful in tracing cell lineage and to produce animal models for certain human diseases.

2.2.2. Vectors for Gain or Loss of Function

To achieve the optimal level of gene expression in specific tissues/cells and at the appropriate time, the choice of promoters used in the construct of a gene-expression cassette is most critical. A specific promoter fused to an

A. Random addition of genetic material by pronuclear injection



B. Gene Targeting

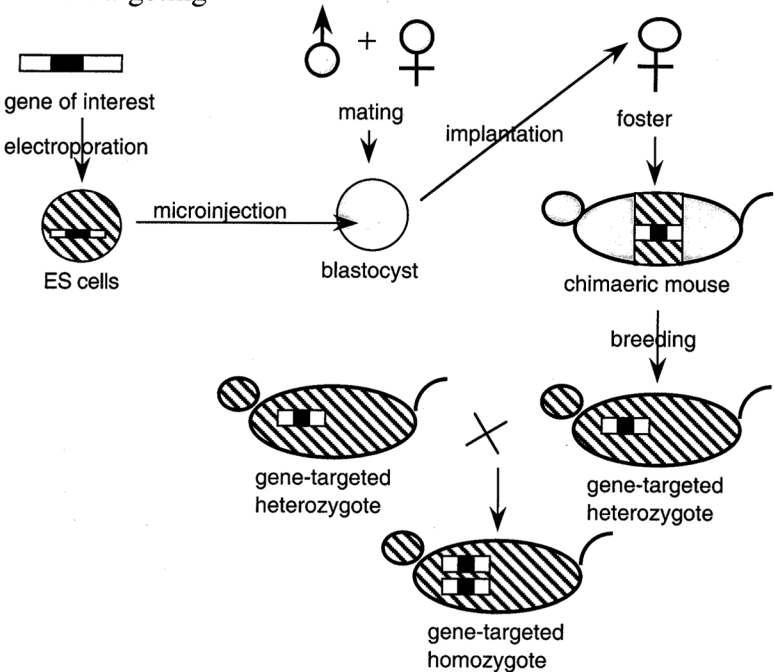
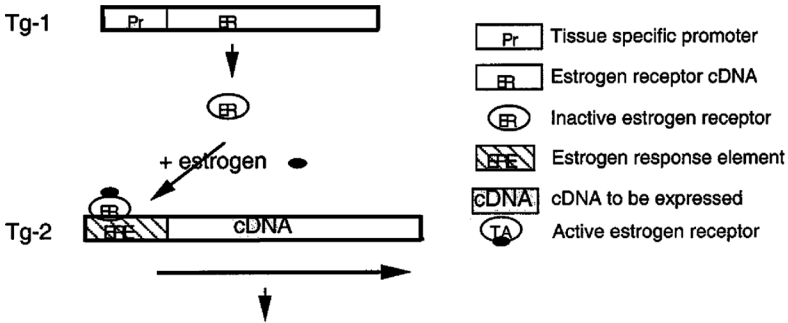


Figure 1 (Caption on facing page)

appropriate translational control element, followed by the addition of a strong polyadenylation signal, such as the SV40 poly(A) site, is essential for successful transgene expression. Addition of an intron also facilitates the expression of transgenes (for reviews, see [Ref. 13](#) and references therein). In the early 1980s, ectopic expression was achieved by employing a ubiquitous promoter, such as the actin or thymidine kinase (tk) gene promoter. Later, tissue- or cell type-specific promoters were used to direct transgene expression in a particular tissue or cell type. Currently, the major limitation in the application of this technology resides at the choice of appropriate promoters or regulatory sequences for engineering the transgene. As such, there is an urgent need to identify regulatory DNA elements that can be used to direct tissue- and developmental stage-specific expression of transgenes.

Improvements have also been made by developing inducible promoter systems. Several inducible systems have been developed, such as the heavy metal-inducible promoter of the metallothionein (MT) gene (14), an interferon-inducible promoter Mx1 (15), the tetracycline-inducible systems (16), and the hormone (estrogen and ecdysone) -inducible systems (17). None of these systems are perfect. For instance, the human MT promoter lacks the desired level of specificity and inducibility by heavy metals (14). The efficiency of the interferon-inducible promoter varies among different tissues due to penetration problems in certain tissues. The tetracycline-, estrogen-, and ecdysone-inducible systems are binary systems consisting of two sets of transgenes ([Fig. 2](#)). In these systems, the expression of a specific cDNA (designated as transgene-2) is regulated by transgene-1 (encoding a transactivator for the promoter directing transgene-2) and the inducer (hormones or antibiotics). The power of these newer inducible systems has been demonstrated in several studies (18,19) and proved to be promising. However, the use of antibiotics and hormones may potentially introduce some unwanted effects. For instance, antibiotics can cause drug

Figure 1 (facing page) Genetic alteration of animal genomes by random addition or gene targeting. (A) Random addition of genetic material (i.e., transgenesis). The recombinant DNA is directly injected into fertilized mouse embryos, which are subsequently transferred into foster mothers. Live born animals from these injected embryos will carry the recombinant DNA in their genomes. (B) Gene targeting of animal genome. The recombinant DNA is first introduced into ES cells by a gene-targeting procedure. The genetically altered ES cells are then injected into blastocysts that are subsequently transferred into foster mothers. Animals derived from these injected blastocysts are made up of two cell populations and are called chimera, one derived from the ES cell pool and the other from the blastocyst cells. By breeding the chimeric animals to a different strain of mice, ES cell genome that carry the mutated gene will be transmitted to the offspring, often heterozygous. These gene-targeted heterozygotes can then be bred to homozygosity.



In the presence of estrogen, the cDNA is expressed in Pr-specific tissues.

Figure 2 A strategy for conducting inducible, tissue-specific expression of a gene of interest in transgenic animals. Transgene-1 (Tg-1) utilizes a tissue-specific promoter-A (Pr) to direct the expression of the transactivator (TA) for the tetracycline operon (Tet-op). Transgene-2 (Tg-2) encodes the cDNA of interest under the control of Tet-op. In the absence of Tet, TA produced in Pr-specific tissues is active, thereby activating expression of Tg-2 in Pr-specific tissues. In the presence of Tet, TA remains silent, no Tg-2 is expressed (25).

resistance and hormones may alter animal physiology that will complicate the interpretation of phenotypes.

In a preclinical setting, transgenic animals are created for testing therapeutic effects and for toxicological studies. In this context, the physiological relevance of these animal models is most critical. Inducible systems are most desirable because the disease state can be optimized by regulating the expression of the transgene. However, the application of antibiotics or hormones to these animals presents a major concern because it may alter animal physiology. Although the insect hormone is generally considered harmless in mammals, recent research on hormones that act on nuclear receptors has suggested a potential interaction of hormonal systems among different animal species (20). Therefore, it remains an active research area to modify these binary inducible systems so that the potential side effects can be eliminated.

2.2.3. Vectors for Examining Regulatory Mechanisms of a Specific Gene

One standard approach to examine regulatory mechanisms for gene expression utilizes reporter gene cassettes driven by the regulatory sequence of a gene under investigation. The reporter cassette usually consists of a cDNA sequence coding for an enzyme or an easily detectable molecule, such as

chloramphenicol acetyl transferase (CAT), β -galactosidase (*lacZ*), luciferase (Luc), or green fluorescent protein (GFP). These reporters share two common features: the enzyme or fluorescence activity is generally lacking in mammals and the activity can be detected by simple assays. The CAT assay was one of the first assays employed; however, this assay is more time consuming and less specific (21). The use of *lacZ* reporter to identify temporal and spatial regulatory DNA elements has proven very powerful (22). For instance, the *lacZ* assay can be conducted in situ to reveal patterns of regulation (23,24) or in a quantitative manner to quantify the level of expression (25). The Luc assay is most sensitive; however, it is not appropriate for in situ detection (26). Green fluorescent protein can be detected in situ in living animals without any incisions of animal tissues (27), which proves to be extremely powerful to uncover changing patterns of gene expression in real time. Furthermore, a number of GFP mutants have been developed that generate different fluorescence signals. This battery of GFPs allows the expression patterns of several genes to be followed simultaneously (28).

Despite the rapid development of the vector systems, the choice of specific regulatory elements for directing transgene expression remains very limited and often becomes a bottleneck in the application of transgenic technology. In the case of preclinical studies where the evaluation of therapeutic agents must be conducted in relevant animal models, the specificity of transgene expression is the primary concern. As such, it is essential to identify specific gene regulatory sequences in the development and application of this technology.

2.2.4. Vectors for Blocking Specific Biological Pathways

Before gene-targeting techniques became widely available, the issue regarding a lack of specific protein function or a specific biological pathway was addressed by employing several less specific strategies, i.e., antisense RNA blockage (29–31), ribozyme blockage (32,33), cell ablation (11,12), and dominant-negative mutation (34), and so on.

The antisense RNA could potentially form a double-stranded complex with the endogenous mRNA precursor, disrupting the function of endogenous transcripts by several mechanisms (30–32). However, lack of specificity is the major problem in antisense approaches. Another RNA-based approach is the use of double-stranded exogenous RNA to ablate specific endogenous RNA species; however, this approach remains at experimental stages (35). Another recently developed approach is the use of ribozyme that seems to have a better efficiency and a higher specificity in cleaving specific endogenous RNA species (32,33). By using appropriate promoters, antisense RNA, double-stranded RNA or ribozyme can be directed to specific cell types, thereby blocking the expression of specific proteins from endogenous mRNAs in these cells. This type of approach has been successfully applied and demonstrated in several studies (36,37).

Specific cell ablation utilizes toxin genes linked to specific promoters, which allows certain cell types to be removed by the action of the toxin. Two most widely used toxin genes are the viral tk gene and the diphtheria toxin A gene (DT-A)(38). To better control toxin expression, an attenuated form of the DT-A, tox-176 gene, has been engineered that appears to be more effective in achieving cell ablation in transgenic mice (39).

Dominant-negative mutations can also be applied to address the function of certain genes. In principle, the information about structural and functional domains of a specific molecule must be first obtained from *in vitro* studies. On the basis of the available information, specific dominant-negative mutations can then be generated and introduced into animals, and the effect of mutation can be addressed in the context of whole animals.

In summary, the goal of changing or blocking specific biological pathways in animals can be achieved by using different approaches. One essential component is the promoter or regulatory element (see the earlier section) to be used, which ultimately determines the specificity of this manipulation.

2.2.5. Introduction of Recombinant DNA into the Mouse Genome

It was first demonstrated by Jaenisch and Mintz in 1974 that foreign DNA, such as SV40 viral DNA, could be introduced into fertilized mouse eggs by infection and subsequently detected in various tissues of the animals that are developed from these embryos (40). Later, a variety of other methods were developed to introduce foreign DNA into fertilized eggs, including infection by retroviruses such as Moloney murine leukemia retrovirus and microinjection. Because of the limited capacity of viral genomes and their preferential integration into “hot-spots,” the procedure utilizing viral infection is less popular. In contrast, direct microinjection of DNA into pronuclei of fertilized eggs is more reliable and has become a widely applied, standard procedure to produce transgenic animals. Details for setting up a microinjection system and maintaining mouse colonies have been described in several laboratory manuals (41,42). In general, the efficiency in the production of useful transgenic animals is determined by several factors. First of all, the design of a given recombinant DNA must meet the need of the experiment (see earlier sections). Secondly, the plasmid sequence must be removed and the DNA to be injected must be of high quality and of appropriate size. The concentration of DNA solution to be injected is also critical. Finally, the strain of animals serving as egg donors is important in terms of the survival rates of eggs following injection and the fostering ability of female animals.

Recently, an alternative method has been reported that makes use of sperm to deliver DNA into the eggs (43). However, the efficiency of this method remains to be confirmed. Currently, microinjection remains the most efficient and effective way to produce transgenic animals of various

species. However, the efficiency appears to be very poor in the management of large farm animals. To produce transgenic livestock, other methods are still being invented and developed. For instance, a method that introduces reverse-transcribed genes into the oocytes during metaphase II has been reported, which greatly improves the efficiency of transgenesis (44).

2.2.6. Analysis of Transgenic Animals

The ultimate goal in the production of transgenic animals is to be able to express a transgene stably in the offspring. The first step is usually to establish several founders. Genomic southern blot or polymerase chain reaction (PCR) analysis of DNA isolated from a small piece of tail sample is commonly used to detect the presence of transgenes in the genomes of the founder animals. These founders, generally heterozygotes, are then bred to nontransgenic animals to establish the lines, and homozygotes are produced by crossing heterozygotes derived from the same lines or back crossing. The expression of the transgene can be analyzed in the founders, the F1 generation or their offspring. Additionally, it is important to confirm the presence and expression of the transgene in offspring of extended generations. The instability of transgenes in heterozygotes is a common problem, referred to as “loss of heterozygosity.” Once the transgene is established in a state of homozygosity, it appears to be relatively stable in most cases (45). Finally, the copy number of transgenes may affect the efficiency of transgene expression, but in most cases, only the copy adjacent to the endogenous sequence is active and the internal copies are methylated and silenced (46).

2.3. Gene Targeting

Gene-targeting experiments allows a wide variety of genetic changes to be introduced into specific genomic loci, ranging from simple point mutations and gene disruption to large deletions and translocations (Fig. 1). This type of experiment requires the perfection of three principal procedures: to design gene-targeting vectors, to manipulate ES cells, and to introduce ES cells into embryos. Despite of a tremendous amount of effort that has been invested in developing ES technology for gene-targeting experiments in many animal species, this procedure is established only for the mouse and the rat at the current time.

2.3.1. Design of Gene-Targeting Vectors

The principle of gene targeting in mammalian genomes was inspired by the observation that homologous DNA recombination occurs in eukaryotes such as yeast. In mammalian cells, homologous recombination occurs spontaneously, but is extremely rare. Without a selection scheme, it is almost impossible to detect this type of genetic event in mammalian cells. Therefore, the most critical factor for successful gene-targeting experiments is the design of targeting vectors that allow efficient selection for homologous

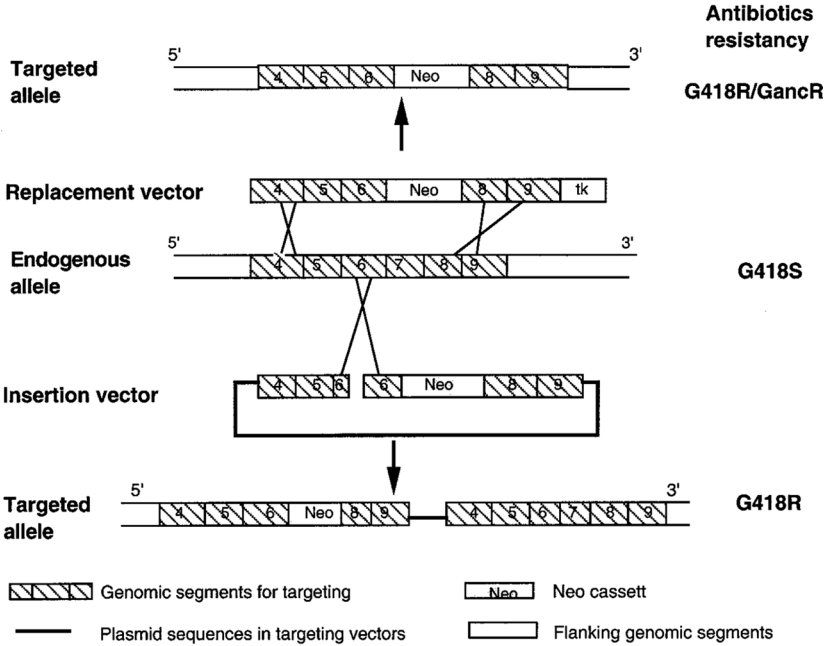


Figure 3 Two prototype gene-targeting strategies. Replacement vector is designed such that homologous pairing between the linearized vector and the endogenous sequence allows two crossing-over events to occur within the two homologous regions flanking a mutated sequence (such as by an interruption with a Neo marker). Thus, the vector sequence replaces the endogenous sequence and renders the recombined allele Neo⁺. The insertion vector is designed such that the continuous genomic segment (e.g., segment 6) is interrupted upon linearization of the vector. With one crossing-over event, the entire sequence is inserted into the endogenous sequence, resulting in the disruption of the continuity of the endogenous sequence (15,47).

recombinants. A mutated DNA sequence is introduced into the targeting vector, which will then be delivered into the genome of ES cells following homologous recombination between the targeting vector and its endogenous counterpart in the ES cells. In theory, it is possible to make changes in all the isolated genes and introduce these mutations into ES cells by using this strategy. The most common kind of mutation is to “knockout” the function of a specific gene. For this purpose, two prototype-targeting vectors are designed, called insertion and replacement vectors (47) (Fig. 3). The insertion vector is more effective in generating homologous recombinants because it requires only one recombination event (47), but the targeted alleles are generally less stable due to the repetition of some sequences.

In contrast, replacement vectors generate more stable homologous recombinants but they occur less frequently. By incorporating a second selection marker, such as the viral tk gene into the replacement vector, and by employing a double-selection procedure, i.e., positive (Neo⁺, G418r) and negative (tk⁻, Gancr or FIAUr) selections (Fig. 3), the frequency of homologous recombinants can be dramatically enhanced (48). Recently, more negative selection markers have been developed, such as a diphtheria toxin DT-A and an immunotoxin gene (49,50). Currently, most gene-targeting experiments utilize replacement vectors and a double-selection strategy. Most animals that have been produced by gene-targeting procedures are “knockout” animals because the targeted gene function is destroyed.

In addition to knocking out a specific gene, attempts have been made to alter genes in a very fine manner, such as to introduce point mutations by using procedures like “Hit and Run” (51) or “In-Out” (52). These procedures require two steps in the manipulation of ES cells. The first is to insert a targeting vector carrying a specifically mutated sequence into the ES genome, which results in a duplication of a portion of the gene. Then, the normal sequence is excised as a result of intrachromosomal recombination, leaving the mutated sequence in the genome. More recently, refined methods that allow gene targeting to occur in a regulated manner have been developed by incorporating a site-specific recombinase system into the gene-targeting procedure (see sections in what follows). In addition to uncovering the function of a specific gene product, a number of biological questions can also be addressed by simple gene-targeting procedures. For instance, the addition of a reporter such as *lacZ* or GFP into the replacement vector allows the expression pattern of the targeted gene to be followed (53,54). For genes that have vital functions, the fate of cells that express this gene can be traced by following the expression of the reporter; therefore, the mechanism underlying the pathological effect of the mutated gene may be explored and the tissues/cells affected by the mutated gene can be identified. Another important application involves the rescue of the mutated gene by “knocking in” a replacement gene (55). The “knockout” and “knockin” combination proves to be a very powerful approach in delineating complementation pathways among gene families.

2.3.2. Manipulation of ES Cells

Embryonic stem cells are generally derived from the inner cell mass of blastocysts and must be maintained as a totipotent cell line in cultures in the presence of a differentiation-inhibiting factor such as leukemia inhibition factor (56). Although different methods are available to introduce DNA (targeting vector) into ES cells, the most widely used method is by electroporation. Technical details in establishing and handling ES cultures have been provided in several laboratory manuals (41,42). Following electroporation, antibiotic-resistant colonies are selected, and genomic DNA from these

clones is analyzed on Southern blots or by PCR to identify clones that have the endogenous gene targeted at one allele. The goal in culturing ES cells for gene targeting is to maintain its totipotency following the electroporation and subsequent cloning/selection procedures, which ultimately determines the chimeric potential of these cells in the recipient embryos. Only the ES cells that can colonize the germ cells will transmit the genetic changes to the offspring. Therefore, germ line chimerism is required to obtain successful gene-targeted animal lines. The factors that affect the efficiency of chimerism remain to be elucidated. These factors include, but are not limited to the following: certain differentiation inhibiting factors provided by the feeders, the source of water, serum, cell line, and the culturing procedure.

An important issue in gene-targeting experiments to produce animal models concerns the genetic background of the animals that will be analyzed. Accordingly, the genetics of ES cells is critical for this type of experiments. For technical and biological reasons, most ES cell lines are derived from a specific mouse strain, 129. As more gene-targeted animals are produced and analyzed, it becomes obvious that strain-specific differences can sometimes affect the phenotypes, complicating the interpretation of data. Recently, ES cells of other mouse strains such as BALB/c and C57/BL6 have become available. For certain diseases, particularly behavioral disorders, it is desirable to examine the patterns of abnormality in several strains of gene-targeted mice or different species such as the rat. Therefore, it is of considerable interest to establish ES cells from other strains of mice and from species other than the mouse.

2.3.3. Introduction of ES Cells into Embryos

It was first demonstrated in 1968 that embryonic cells could be isolated from early embryos and introduced into blastocyst- or morula-stage embryos of different genetic backgrounds. These embryos could then develop, following implantation in the foster mothers, into chimeric mice composed of two cell populations, one derived from the recipient embryos and the second from the injected cells (57). Later, it was found that ES cells derived from early stages of embryos (58,59) could maintain their pluripotential in cultures, and the culture-derived stem cells could integrate into the ES-cell pool of the recipient embryos and populate various tissues of the chimeric conceptus and adult (60,61). From the chimeric mice, the germ cells which were derived from the ES cells could be fertilized and developed into animals that carried one complete set of ES chromosomes in their genomes. Therefore, it is possible to manipulate ES-cell genome *in vitro* and subsequently introduce the manipulated ES cells into recipient embryos for the production of chimeric mice. Technically, the most critical criterion for a good ES-cell line is its potential to colonize the germ-cell lineage in the recipient embryos (60). Currently, many ES-cell lines have been established, but only a few have been demonstrated success in generating germ line chimera. Most ES cells

require feeder cells, but ES-cell lines that are able to maintain their totipotency without a feeder layer have also been established (62).

Two methods have been developed to introduce ES cells into recipient embryos in the mouse: aggregation and blastocyst injection (63). However, most studies rely on blastocyst injection because the success rates of the aggregation method are highly variable among individual laboratories. Currently, the blastocyst-injection procedure is used routinely in most laboratories that conduct gene-targeting experiments. Technical details are also available in several lab manuals (41,42).

The first successful demonstration of germ-line chimerism using a modified aggregation method to aggregate ES cells with recipient embryos was reported in 1993 (65). Subsequently, this technique was under intensive experimentation to improve its consistency and efficiency in many laboratories. Complete ES-derived mice were produced later using this method (64). Although this method requires no injection station and appears to be simpler, it demands highly trained personnel and the efficiency is not as consistent. Currently, most laboratories rely on blastocyst injection to generate gene-targeted mice.

2.3.4. Analysis of Gene-Targeted Animals

Unlike the analysis of transgenic animals with random addition of DNA in which transgenes can be detected in the founder animals (usually heterozygote) and germ-line transmission can be confirmed in the first generation of the offspring derived from the founders, the analysis of gene-targeted mice requires an additional step. The offspring derived from the injected blastocysts are chimeric animals, which are first identified on the basis of coat-color chimerism (*agouti* for most ES cells, which is different from recipient embryos). To obtain heterozygote founders, these chimeric mice have to be bred with different strains of mice such as BALB/C or C57BL/6 in order to obtain animals inheriting the ES genome in the heterozygotic state. Once germ-line transmission of the ES genome is confirmed (i.e., the chimeric mice are able to produce heterozygotes that inherit the ES genome carrying the mutated gene), a breeding program is started to produce homozygote animals with both alleles of the endogenous gene mutated. The phenotype of gene-targeted animals is generally analyzed in the homozygote animals. However, dominant-mutant phenotypes of genes that are located in the sex chromosomes can be identified at the heterozygotic stage. By definition, gene-targeted mice are also “transgenic” mice, even though the transgenic material is more of a replacement than an addition to the genome, as seen in transgenic mice generated by pronuclear microinjection. Because gene targeting provides a much more specific and controllable tool to manipulate animal genomes, it is desirable to conduct this type of experiment in other animal species, such as the rat or other livestock, which may be more appropriate animal models for human diseases. However,

ES-cell technology is available only for the mouse and the rat at the current time, despite an enormous amount of effort to develop this technology in other species. Currently, thousands of gene-targeted mouse lines have been generated and documented (for reviews, see [Refs. 65–67](#)).

Although transgenic mice generated by pronuclear DNA injection are not perfect animal models, they can be suitable to address certain biological questions. Furthermore, the inducible gene-targeting strategies require the use of transgenic mice to introduce a recombinase system into the gene-targeted mice (see in what follows). Therefore, current progress in this technology includes experimental designs for both pronuclear DNA injection and gene-targeting procedures.

2.3.5. Pitfalls in Gene Targeting

Although gene targeting can be very specific and animals produced by this approach can be valuable tools for preclinical studies, several potential pitfalls have drawn the attention of scientists in the field as more animals are produced and analyzed.

The first and most common pitfall is incomplete knockout of the gene under investigation. When the targeting event does not totally inactivate the activity of the targeted gene, the phenotypes may not be explainable. This can result from exon skipping, read-through transcription/translation, or the use of alternative/cryptic promoters (68–72).

The second pitfall is the generation of truncated or mutant forms of the gene products that may still function in certain pathways, complicating the knockout phenotypes. For instance, a GABAA, $\alpha 6$ -knockout mouse model is complicated by subunit interference as a result of generating a truncated $\alpha 6$ subunit in these mice (73).

The third pitfall is associated with interference from other genes that are linked to the mutated gene. For instance, a regulatory sequence may be shared by several genes. Disruption of this sequence will result in removal of not only the gene under investigation, but also as yet unidentified genes. Complication caused by deleting a regulatory sequence shared by several loci has been reported in several studies such as a myogenic regulatory factor 4 knockout (74) and a T-cell receptor component CD3- η knockout (75). Conversely, a strong promoter, commonly used to drive the expression of selection markers, may activate the expression of neighboring genes. This type of problem has also been reported in several studies including knockout of a locus control region of the β -globin gene (76), and the *Hoxd-10* (77). In order to address this concern, it is now widely accepted that selection markers should be removed whenever possible. This can be accomplished by flanking these markers with a pair of *loxP* sequences (see in what follows).

The fourth pitfall concerns the strain-specific factors that may influence the penetration of phenotypes. For instance, reduced kainate

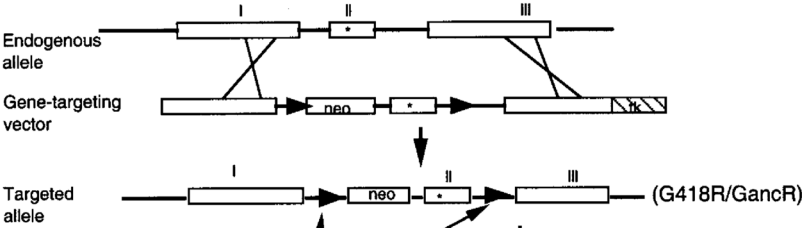
sensitivity in p53 knockout hybrids of 129/SvEMS $\times$ C57BL/6 (78) has been challenged by the observation that C57BL/6 wild-type mice are already resistant to kainate (79). By introducing the identical mutation into several different genetic backgrounds, this type of pitfall can be minimized. Finally, gene-redundancy and compensatory mechanisms may complicate the interpretation of phenotypes. Many gene knockout mice exhibit no obvious phenotypes, presumably due to the action of redundant genes. In addition, certain early defects caused by the knockout may be masked by the induction of compensatory pathways in the adults. Accordingly, functional redundancy appears to be a common problem, especially for those genes that are involved in vital functions.

2.4. Inducible Gene-Targeting

Gene-targeting procedures allow the introduction of genetic changes at specific genomic loci, which circumvent many problems associated with random DNA integration. However, once a mutation is introduced into the genome by gene targeting, it remains permanent in the manipulated animal genome, which may cause embryonic lethality due to the lack of a vital function of the specific gene or may drastically affect animal physiology as a result of compensatory mechanisms in the mutant animals.

To solve these problems, regulated or inducible gene-targeting procedures have been developed, which allow specific mutations to be introduced and rescued in a regulated manner. This is made possible by the introduction of a second system, the site-specific recombinase that dictates the temporal and spatial specificity of the gene-targeting event. Two site-specific recombinase systems have been developed: a yeast recombinase coupled with its recombination targets (80,81) and the P1 phage recombinase Cre coupled with its target, a 34-bp long partially palindromic sequence called *loxP* [*locus* of crossover *x* in P1 (82,83)]. The Cre-*loxP* system is more efficient and consistent in inducing homologous recombination events and is applicable for larger DNA fragments. Figure 4 illustrates an example of using Cre-mediated recombination to delete exon II of a gene of interest in specific tissues. Once the *loxP* site(s) is(are) inserted in specific genomic loci by gene-targeting procedures, a wide variety of genetic engineering can be conducted by introducing the Cre enzyme. The Cre enzyme can be introduced at the ES stage or carried in a transgenic mouse under the control of a specific regulatory sequence. To produce more aggressive genomic alterations, the *loxP* sequences can be introduced into loci that are far apart or even on different chromosomes. Depending upon the orientation and copy number of the *loxP* sequences, chromosomal deletions, inversions, duplications extending to 3–4 cM, and translocation are possible (84).

A. Gene targeting to replace the sequence to be deleted with a segment flanked by a pair of loxP boxes



B. A Cre enzyme expression cassette under the control of a specific promoter



C. Deletion of exon II via recombination between loxP sites mediated by Cre expressed in specific tissues

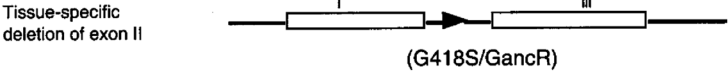


Figure 4 (A) Tissue-specific gene targeting. The endogenous sequence to be deleted is first modified by a gene-targeting event, resulting in the sequence flanked by a pair of *loxP* sequences. The gene remains normal because no transcribed sequences are deleted or mutated. (B) A transgenic mouse line is produced, carrying the Cre enzyme under the control of a specific promoter. (C) By breeding the two transgenic mice (from steps A and B), the offspring animals express Cre in specific tissues, rendering segment * deleted in these tissue as a result of site-specific recombination at the two lox sites (step D) (62,83).

2.5. Addition of Genetic Material into Somatic Cells (Gene Therapy)

In order to deliver biological products or genes into humans as a therapeutic agent, such as in gene therapy, it is desirable that genetic changes be made only in the somatic cells. Animal models for preclinical tests of these types of agents are essential as the pharmacological effects and toxicity of these agents must be evaluated in the context of whole animals. The most relevant animal models are those in which genetic alteration of somatic tissues is used to approximate the human condition. Additionally, for certain therapeutic

agents that target at specific receptors, it will be desirable to use animals carrying the corresponding human receptors for preclinical evaluation. For instance, a human cytokine-expressing vector should be tested in animals that carry the corresponding human cytokine receptor in appropriate tissues. To meet such a need, the gene for human cytokine receptor will be expressed in transgenic animals. To introduce changes into either somatic tissues or germ cells, all three types of genetic changes described earlier (i.e., random DNA integration, gene-targeting and inducible gene-targeting) can be applied. The challenge is the selection of appropriate expression vectors and regulatory sequences, obtaining appropriate cell types (in the case of somatic changes) for manipulation and the delivery methods. This remains an active research area and requires further exploration. These novel therapeutic agents/methods utilize cellular products or ex vivo manipulation of cells followed by reintroduction of these cells into humans. Specific concerns in the use of these types of therapeutic agents may vary from agent to agent; however, general concerns such as cell types, gene products, and vector systems are common to this type of genetic manipulation. Although many remain at the experimental stages, it can be predicted that the number of preclinical studies will grow rapidly, and the demand for animal models will increase dramatically. New guidelines for these types of therapeutic agents/methods are available at the Web site of FDA "<http://www.fda.gov.cber.guidelines.htm>."

3. APPLICATIONS IN PRECLINICAL DRUG DEVELOPMENT

Essentially, all studies in preclinical drug development involve animals. Two major goals in animal studies of preclinical drug development are to confirm the pharmacological effects of the drug and to determine and explore its potential toxicity in the context of animal physiology before a human trial is launched. The most elegant application of transgenic animals to evaluate the pharmacological effects of drugs is to create suitable disease models. The therapeutic value of the agent and its metabolism can be determined in these genetically altered animals that are relevant to human conditions. In preclinical studies of drug toxicity, transgenic animals are generated for screening general toxic effects and mutagenic/chronic effects. Additionally, transgenic animals can be generated in which a specific human condition associated with the therapy can be simulated in the animals. The pharmacokinetics and pharmacodynamics of these drugs in these types of patients can be assessed first in these animals. In the following, the application of transgenic animals in preclinical drug development is discussed with respect to biological activity, drug metabolism, and toxicity/carcinogenicity.

3.1. Disease Models for Evaluating Biological Activities of New Drugs

A prerequisite of utilizing transgenic animals for establishing disease models in which a new drug is to be tested is the identification and cloning of the genes that are potential targets of the drug or are involved in the signaling pathways of the actions of the drug. This strategy is applicable in many human diseases, especially in those conditions where the diseased gene(s) are known. The most intensively investigated areas of research for preclinical/clinical application of transgenic technology are neurological/behavioral disorders, cardiovascular disorders, cancers, and immune disorders.

3.1.1. Disease Models for Neurobiological/Behavioral Disorders

The primary targets for drugs that affect cognition and behavior are neurotransmitters and the receptors or components involved in their effector systems. In this area, numerous disease models have been generated by transgenic technology for preclinical testing. For instance, several transgenic mouse models of amyotrophic lateral sclerosis (85) have been established by expressing a mutant human superoxide dismutase gene that enhances the generation of damaging oxygen radicals. A transgenic pig model for retinitis pigmentosa has been established by expressing a mutated rhodopsin gene (Pro34Leu) (86). Like human patients, these pigs have early and severe rod loss and provide a large animal model for preclinical tests. Alzheimer's disease (AD) is a common disease in the aging population. Alzheimer's disease causes a progressive intellectual failure and affects life quality dramatically. A large volume of studies has been conducted in both basic and clinical settings, both suggesting a primary pathologic role of mutations in the β -amyloid precursor protein (APP) in the manifestation of this disease (87). The first transgenic model of AD is a transgenic mouse model that expresses a mutant APP and progressively develops many of the pathological hallmarks of AD (88). Currently, a number of transgenic models have been produced (89–92) and drug screening/testing programs using these animal models have been launched (93,94).

Neuropsychology and behavior are generated by collections of neural components that constitute a system requiring both input and output properties. As such, the molecular etiology of psychological and behavioral disorders remains poorly understood and the treatment is mostly symptomatic and far from satisfying. Recent molecular genetic studies, especially the application of targeted mutations, have begun to uncover certain genes or gene products that may be involved in behavioral disorders. Genetically altered animals that exhibit behavioral abnormalities have been produced by altering the expression/synthesis of neurotransmitters, their receptors, the components of the signal transduction pathways, and so on (95,96).

However, one must be cautious in the interpretation of the overwhelmingly interesting behavioral abnormalities of these mutant animals (97). Many of these mutant mice exhibited phenotypes that could not be satisfactorily interpreted based upon our current knowledge about these molecules. Nevertheless, this technology has progressed tremendously over recent years and will continue to be improved. The ability of inducing molecular mutations and ablating specific cells in a temporally and spatially specific manner opens the door to a plethora of strategies aimed at behavioral modification. As it becomes feasible to introduce very specific mutations, both temporally and spatially, it will be possible to create animal models in which the component specificity of the lesion, as well as the behavioral specificity following the lesion, can be addressed. In the near future, animal models reflecting a specific disease state and bearing a physiological relevance to a particular human behavioral/psychological disorder will become available once a potential candidate gene is known.

3.1.2. Disease Models for Cardiovascular Systems

Most molecular targets of drug therapy for the cardiovascular system are well understood, such as the renin/angiotensin system (RAS), the β -adrenergic receptor (β -AR) system, the ion channels, and lipoprotein metabolic pathways. The genes for components in the RAS, β -AR systems, and lipoprotein metabolic pathways are better characterized; hence, more transgenic animals have been produced for testing drugs targeted at these components. In contrast, the genes for ion channels are only beginning to be disclosed and fewer animal models are available. With respect to cardiovascular physiology, the rat is more relevant to the human; therefore, transgenic rat models are more desirable. However, due to technical difficulties and housing problems, the vast majority of transgenic animal models for cardiovascular disorders are made in the mouse. The first attempt was made by expressing the rat and human renin and angiotensinogen genes in transgenic mice (98,99). In these animal models, species specificity of renin substrate was demonstrated, which allowed renin inhibitors to be tested and the enzyme kinetics to be conducted in vivo. A tremendous amount of effort was invested to conduct "gene targeting" in rats, this technology as now been established. Mutant mouse lines with defects in several of these genes have been created by "gene-targeting" procedures, such as angiotensin-converting enzyme-deficient mice (100) and the angiotensin II type-2 receptor-targeted mice. Another important cardiovascular component is the β -AR system, which represents the primary myocardial targets of the neurotransmitters, norepinephrine and epinephrine. For therapeutic agents that target at this receptor system, several transgenic mouse models were made by using tissue-specific overexpression of the components of the adrenergic system, such as the β 2-AR (101), the α 1 β -AR mutant (102), a β -AR kinase (β -ARK) and a β -ARK inhibitor (β -ARKI) (103). The β 3-AR transgene

expression resulted in enhanced myocardial function, providing an experimental basis for gene therapy in the case of heart failure. The transgenic β -ARK and β -ARKI studies demonstrated a role of this kinase system in heart disease. Mutant mice deficient in these components have also been generated, such as the α 3-adrenergic receptor (104). These animal models provide valuable tools in preclinical testing of drugs acting on these targets.

The lipoproteins are responsible for transporting plasma cholesterol and triglycerides, thereby regulating cholesterol homeostasis. Transgenic animal models for a diseased lipoprotein system fall into two categories: models of altered receptor function and models of altered production of lipoprotein ligands (for reviews see Refs. 105, 106, and references therein). Transgenic animal models with altered low-density lipoprotein receptor (LDLR) expression have (LDLR) expression have provided new insights into the role of LDLR in the clearance of lipoprotein. Furthermore, studies of transgenic mice with abnormal expression of lipoprotein ligands indicated multiple gene interactions in the regulation of HDL. These animals are potential models for preclinical testing of drugs that lower plasma concentrations of lipoproteins by either lowering their production or stimulating their removal.

3.1.3. Disease Models for Cancer

The battle with cancer involves the entire biomedical research community in a multifaceted fashion. By using genetic approaches, our understanding of the underlying mechanisms for cancer formation, such as cell division, differentiation, migration, and death, has advanced dramatically. Particularly, transgenic animal models permit the sequential dissection of complex oncogenic processes in the context of animal physiology. In the drug industry, the most exciting advancement is the development of novel therapeutic strategies for treating cancers and the re-evaluation of conventional therapeutic agents by using transgenic animal models.

One advantage of using transgenic animal models for cancers is the ability to test novel therapeutic agents or strategies, such as immunotherapy and gene therapy. Transgenic animals can be made to express tumor-specific human antigens, such as transgenic mice bearing the tumor markers carcinoembryonic antigen and germ cell alkaline phosphatase, and mice carrying ectopically expressed oncogenic proteins (107). These transgenic mice express human gene products from embryonic stages, providing ideal models for testing immunological therapies without the complication of host immune responses as frequently seen in conventional animal models bearing tumor grafts. Transgenic mice deficient in tumor suppressors or carrying dominant-negative mutations of these genes are also available for modeling a variety of cancer (108) such as breast cancer (109–111), prostate cancers (112–114), leukemia (115,116), and colon cancer (117). These animal models

are powerful tools for investigating a variety of novel lead compounds. Another approach that utilizes transgenic animals for anticancer drug development is to test the efficacy of novel biological agents in the management of cancer. For instance, the potential of cytokines in cancer management is enormous because of their role in immunity, inflammation, and tissue repair; hence, the use of cytokines in cancer therapy has been rapidly developed. In certain gene therapy protocols, a continuous, targeted, high-level expression of cytokines in the animals is made possible by using transgenic technology (118). In this regard, transgenic animals not only serve as the test tubes to evaluate the agents, but also play a key role in optimizing the dose and schedule.

3.1.4. Disease Models for Immunological Disorders

The most elegant application of transgenic technology to understand the molecular basis of biological processes is in the field of immunology. Not only have the mechanisms underlying the complicated humoral and cellular immunity been delineated in great detail, but also valuable animal models for immunological disorders have become available. For instance, animal models are available for several autoimmune syndromes such as systemic lupus erythematosus (119), autoimmune arthritis (120,121), and virus-induced autoimmune diseases (122). Animal models are also valuable for vaccine development against hepatitis B virus (123), *Helicobacter pylori* (124), and for testing therapeutic intervention of certain immune-related neuron degeneration (125) and dermatological disorders (126). The most relevant preclinically application is the production of animal models for predicting hypersensitivity reactions (127). Medical product-induced hypersensitivity reactions are the relatively common problems, but few predictive models are available because of our limited understanding of the mechanisms and the lack of animals that can be used to predict human immune responses. The use of novel animal model models created by transgenic technology is the most promising avenue for solutions to these problems.

3.2. Animal Models for Studying Drug Metabolism and Disposition

Pharmacokinetic and pharmacodynamic studies are required for most preclinical evaluations in drug development (see [Chapters 4–6](#)). Although studies conducted in normal animals are generally satisfactory, it is often desirable to evaluate drug metabolism in diseased states. For those compounds that are extensively metabolized, physiological changes caused by certain diseased conditions often change the pharmacological characteristics of these compounds. For instance, serum alpha-1-acid glycoprotein (AAG) levels are elevated in several disease states such as depression, arthritis,

severe burns, and autoimmune deficiency. The effects of the elevated serum AAG on drug disposition of imipramine, prazosin, lidocaine, and fluoxetine have been examined in transgenic mice expressing elevated AAG, which provides insights into how disease states may affect metabolism of these drugs, and hence their efficacy as therapeutic agents (128,129).

For those drugs that target at receptors specific to humans, transgenic animals expressing the humanized receptors are essential for testing not only their biological activity, but also their disposition as a result of receptor–ligand interaction. For instance, IDEC-CE9.1 is a macaque/human chimeric antibody that is being developed to manage rheumatoid arthritis (130). This antibody is highly specific to the human T-lymphocyte receptor, CD4; therefore, it is difficult to predict its clinical behavior in traditional animal models that express no human CD4. By engineering transgenic mice bearing human CD4, it is possible to create animal models suitable for evaluating its therapeutic activity and pharmacokinetic behavior (131). It can be predicted that as more biological molecules are developed for therapeutic application, appropriate animal models that are relevant to human conditions will be required for pharmacokinetic and therapeutic studies of these agents. These animal models can be created most efficiently by using transgenic approaches.

3.3. Animal Models for Evaluating Drug Toxicity/Carcinogenicity

Toxicological studies are also essential for preclinical tests. In most cases, animal models are required for safety assessment. The application of transgenic animals in toxicology has been reviewed ((132,133), and references therein). In general, animal models are created to accomplish the following objectives: establish a safe starting dose (and subsequent escalation scheme), identify potential target organs of toxicity, determine potential genotoxicity, and select parameters for monitoring toxicity. Among these goals, a comprehensive assessment of the genotoxic potential in the context of whole animals is most challenging because of the time required to obtain data points by using conventional animal models. Traditionally, a battery of standard genotoxicity tests include an initial *in vitro* test in bacteria, such as the Ames assay, an assessment of gross chromosomal damage in mammalian cells such as CHO cells, and finally an *in vivo* test for genetic damage. However, in transgenic animals that harbor an easily detectable reporter, such as *lacZ* in shuttle vectors that can be recovered from the mammalian genome for quantifying the mutation rates, the assessment of mutagenic potential *in vivo* can be greatly simplified. Several established mutation assay systems such as the MutaTM mouse (134) and Big BlueTM (135) have been the subject of several reviews (136–140). These models carry the insertion of a bacterial gene, either the reporter *lacZ* gene or the *lacI* repressor gene acting on *lacZ*, in the genome, which serves as the target for mutations.

Studies have been conducted to compare conventional *in vivo* genotoxicity tests (e.g., the cytogenetic assay in bone marrow or peripheral blood) with these animal models. It appears that data collected from transgenic animals, in these cases, are more relevant to an assessment of possible risk in man (141).

Another application of transgenic animals in safety assessment is the evaluation of drugs derived from biological molecules such as interferons and recombinant immunoglobulins. Because of the nature of these molecules, the host immune response-mediated pathogenicity is the primary concern when they are to be used for therapeutic purposes. In this case, transgenic animals are made to express these biological molecules for a comparison to normal animals in order to assess the immunopathogenic responses (142,143).

4. THE PROSPECTIVE

4.1. Technical Development

The basic tools to mutate a mouse genome, such as pronuclear DNA injection, blastocyst injection, and vectors for gene targeting, are all well established. Two potentially problematic aspects of this technology stem from the following facts: it is very time consuming to establish targeted ES clones and generate chimeric mice, and useful ES cells are available only from the mouse and the rat. For the first problem, the aggregation method to introduce ES cells into embryos provides an alternative; however, this procedure requires fine tuning. Recent development in generating ES banks representing a large number of gene-targeting clones can potentially shorten the time required for ES work (144). However, the remaining challenge is the connection of each targeted gene to a special disease condition, which requires enormous amounts of basic research. In order to address the second problem, it is necessary to establish ES cells from a variety of animal species that are capable of colonizing gonads efficiently. This problem also requires an enormous amount of effort in research to understand the mechanisms underlying germ cell development.

The design of experimental procedures that allow different genetic tools to be used in combination can also be improved. For instance, the combined use of two transgenic systems, one with the *Cre* gene under the control of a specific promoter and the other with targeted delivery of the *loxP* sites, allows various types of specific gene alterations to be constructed in animals. The level of specificity and the magnitude of alteration can be improved tremendously by careful design. A critical factor is the identification of tissue- and cell-specific regulatory elements for a wide variety of conditions. This essential piece of information awaits more effort in basic biological research. Finally, one should not ignore the complexity of

mammalian organ systems in the interpretation of data collected from these animals; animals do not passively receive experimental manipulation and endogenous gene activities may be altered in response to the introduction of exogenous DNA sequences. Therefore, in addition to technical advancement, additional basic information regarding the control of specific gene expression in animals is required in order to achieve the highest level of specificity and precision in transgenic animal experimentation.

4.2. Application of Transgenic Technology in Drug Industry

Transgenic animals are produced using molecular genetic techniques to add functional genes, to alter gene products, to delete genes, to insert reporter genes into regulatory sequences, to replace/repair genes, to make tissue/lineage-specific alteration of gene expression, and so on. These genetically altered animals provide unique tools for studying a wide range of biomedical problems *in vivo*, allowing the manifestation of specific genetic changes in a variety of biological systems to be examined. Drug development relies largely on animal systems, and several areas of research have been hindered for decades due to the lack of suitable animal models. Established transgenic techniques undoubtedly have facilitated the progress of animal experimentation for some pharmacological questions where potential target molecules have been characterized. Because this technology is evolving rapidly, it is foreseeable that future development of this technology will allow one to address some difficult pharmacological questions where no specific targets have been identified.

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Pharmacokinetics/ADME of Small Molecules

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1. INTRODUCTION

A major objective of the drug development process is to establish that the investigational new drug candidate is both efficacious and safe for human use. Besides pharmacological properties, the efficacy and toxicity of a drug depends upon its pharmacokinetic (PK) properties. Pharmacokinetics can be simply defined as the science that describes the time course of drug disposition including its absorption, distribution, metabolism, and excretion (ADME) properties, following administration of drug.

Pharmacokinetics can be considered a biomarker of drug exposure as well as a marker of efficacy and safety of drugs. It has been estimated that about 40% of drugs fail during development due to inappropriate pharmacokinetics (PK) in humans (1–3). This is very critical because a drug with poor PK properties can be terminated early in development before significant futile investments are made. Therefore, extensive efforts have been devoted to predict human pharmacokinetics earlier during preclinical drug discovery and development, to reduce the occurrence of failures after the drugs are introduced into humans. Efforts have focused on in vitro and in vivo allometric scaling ([Chapter 2](#)) as well as specific comparisons of the individual physiological systems (4,5). Although some progress has been

observed, the accurate prediction of human pharmacokinetics is complicated by interspecies differences in ADME mechanisms.

The pharmacokinetic profile of a compound can be influenced by a number of factors including the physicochemical properties of the drug (6–10), the nature of the drug delivery system (11), physiologic factors (12), environmental factors (13–16), and the presence of underlying disease (16–18). In this chapter, the pharmacokinetic mechanisms (ADME) involved in the disposition of small molecules, and the factors that influence these processes, are presented. For a discussion of ADME processes for large molecules (e.g., proteins), the reader is referred to [Chapter 5](#).

2. PHARMACOKINETICS: GENERAL OVERVIEW

As stated above, pharmacokinetics involves the study of drug absorption, distribution, metabolism, and excretion. Typically, the pharmacokinetic profile of a compound is characterized by measuring the rate of change of drug concentrations in plasma over time following drug administration. Additional information is obtained by monitoring the excretion rate of drug in the urine.

The pharmacokinetic parameters that are commonly measured include the following:

- $C_{\max}$: the maximum concentration of drug in the plasma,
- $t_{\max}$: the time at which the maximal concentration is observed,
- AUC: area under the curve, area of the plasma concentration–time profile from time 0 (when dose is administered) to time ∞ (when dose is completely eliminated),
- V_D : volume of distribution, an indicator of the extent of distribution of a drug in tissue,
- Cl: clearance, an indicator of the rate at which a drug is removed from plasma,
- $t_{1/2}$: half-life, the time it takes for plasma concentrations to decline by 50%, and
- F : bioavailability, the fraction of administered dose reaching the systemic circulation.

The utility of these parameters is based on the concept of linear pharmacokinetics. The underlying assumption of linear pharmacokinetics is first order elimination; that is, the rate of drug elimination from the body is proportional to the plasma concentration. Accordingly, $t_{1/2}$ is constant (dose-independent), and plasma concentrations and AUC are proportional to dose (since V_D and Cl are also assumed to be constant). While linear pharmacokinetics can be applied in most situations, many drug disposition mechanisms (e.g., active transport, drug metabolism) are saturable.

Therefore, in some cases, pharmacokinetics is nonlinear, making dose selection difficult.

Almost 10 years ago, Benet (19) identified the important pharmacokinetic and pharmacodynamic parameters that should be obtained during both preclinical and clinical development. These parameters were ranked (in order of importance) as follows: clearance, effective concentration range, extent of availability, fraction of the available dose excreted unchanged, blood/plasma concentration ratio, elimination half-life, toxic concentration, extent of protein binding, volume of distribution, rate of availability. Therefore, the most important determinant of drug disposition is clearance. Since clearance is the parameter used to establish a suitable dose for a patient, intersubject variability in clearance (due to genetic differences, disease, or drug interactions) can significantly impact pharmacotherapy.

3. MECHANISMS OF SMALL MOLECULE ABSORPTION

When a compound is administered intravenously, the dose is delivered directly into the systemic circulation. All other routes of administration are collectively termed extravascular routes (e.g., oral, nasal, rectal). Following extravascular administration, drug must be absorbed into the bloodstream across a membrane before it is available to distribute to its site of action. For this reason, the bioavailability of the drug is an important issue for drug development. Here, both the physicochemical properties of the drug and the performance of the delivery system influence drug absorption. The impact of formulation and route of administration on drug absorption is the focus of [Chapter 7](#). Presented in this chapter are general mechanisms of drug absorption, with particular focus on oral drug delivery.

3.1. Passive Absorption

The traditional view of oral drug absorption is that it occurs primarily from the small intestine and proceeds via a passive transcellular process. The small intestine represents the primary site of absorption in the GI tract because of the functional specialization of the intestinal cells (creating a large surface area for absorption) combined with the prolonged intestinal transit time.

Passive absorption is usually described by Fick's Law, with the driving force being the concentration gradient across the membrane (20). Passive absorption is governed by several physicochemical properties including solubility, permeability, pK_a , lipophilicity, stability, and integrity, each of which can influence drug absorption and pharmacokinetics (10). Lipinski (6) formulated the "Rule of Five," based on an evaluation of more than 2000 compounds from the World Drug Index. According to this rule, the following properties are ideal for drug absorption, for an ideal drug-like

properties: (1) molecular weight <500 , (2) $\text{Log } P < 5$, (3) sum of hydrogen bond donors <5 , and (4) a sum of hydrogen bond acceptors (as a sum of N and O) <10 . However, the two most important factors regulating oral absorption of drugs are solubility and permeability.

Solubility and lipophilicity are inversely related; that is, a poorly soluble compound is usually a highly lipophilic compound. On the other hand, the ionization state of a solute can influence its permeability across the biological membranes. $\text{p}K_a$, the ionization constant, provides important information on ionization state of a compound across the pH gradient. The pH throughout the GI tract varies considerably [(13); Table 1] and the degree of ionization can influence drug absorption differently at different sites of GI tract. According to the Henderson–Hasselbach equation, a compound will be 50% ionized when $\text{pH} = \text{p}K_a$. For basic compounds, the ratio of ionized:unionized (BH^+/B) increases, at lower pH. Conversely, the ratio of ionized:unionized of a weak acid (A^-/AH) increases as the pH is increased.

Among the physiologic factors that influence oral drug absorption, gastric emptying is perhaps the most important (13). Gastric emptying time (GET) is the time it takes for the stomach contents to empty into the intestine. The phenomenon of GI motility has been extensively studied, and a number of factors including physiological (e.g., stomach content, pH, volume, osmolarity, viscosity, temperature), and nonphysiological (exercise, body position, medications, age) affect this process (13,21). Perhaps the most important determinant of gastric emptying is food. In the fasting state, gastric emptying is a rapid process (repeat cycles of 1–3 hr). However, in the presence of food, gastric emptying slows down significantly (resulting in $\uparrow$ GET up to 9 hr). Therefore, GET can, in certain cases, be the rate-limiting step to absorption. For this reason, clinical studies of oral drug absorption must control the food intake of subjects because the presence of food, the type of food (hot vs. cold meal, liquid vs. solid), and the amount of food can affect GET.

How does food affect oral drug absorption? In general, food decreases the rate of drug absorption by delaying gastric emptying ($\uparrow$ GET).

Table 1 Regional Differences in pH and Residence Time Along the Gastrointestinal Tract: Fasted Conditions

Region	pH	Residence time (hr)
Stomach	1.5–2	0–3
Duodenum	4.9–6.4	3–4
Jejunum	4.4–6.4	3–4
Ileum	6.5–7.4	3–4
Colon	7.4	Up to 18

Therefore, drug absorption proceeds at a faster rate in fasting compared to fed conditions (13). There are, however, conditions where food may also affect the extent of absorption (i.e., the bioavailability). Examples include the following (22):

- *Acid-labile compounds* (e.g., penicillin, omeprazole). These medications degrade in an acidic environment. Therefore, when taken with food, they are retained in the stomach (pH 1–3) longer, resulting in increased degradation and reduced extent of absorption (23,24).
- *Poorly soluble compounds*. Meals increase the absorption of drugs that are primarily absorbed in whole small intestine due to their increased solubility by gastric contents, biliary secretions (e.g., carbamazepine, diazepam, dicumarol, griseofulvin, phenytoin, tocoferol) and fat in the food (e.g., acetaminophen, diltiazem, disopyramide, nicardipine, ranitidine, sumatripan). It is interesting these effects are also related to their biopharmaceutical classification (see below).
- *Tetracycline (TCN) and dairy products*. Calcium chelates TCN and prevents absorption (25).
- *Grapefruit Juice: inhibits intestinal cytochrome P4503A4*. This results in reduced presystemic metabolism of CYP3A4 substrates, thereby $\uparrow F$ (26).
- *Medications with a “window of absorption.”* These compounds are absorbed at specific localized regions of the intestine. Delayed stomach emptying may increase the extent of absorption of these medications by increasing contact time with these localized absorption sites.
- *Propranolol: $F \uparrow$ with high protein meal*. This results in reduced first pass hepatic metabolism by increasing splanchnic blood flow (27).

In some cases, a delay in drug absorption in the presence of food may be disadvantageous due to the resultant delayed onset of therapeutic effect, as when a medication is administered with a meal. An additional concern, however, is the dose-dumping phenomenon noted extended release formulations of compounds when given concomitantly with food. This is particularly important for medications with a narrow therapeutic index (e.g., theophylline) (28). In these cases, food may result in an unintended rapid release of the drug. The theophylline experience certainly sensitized the regulatory agencies, and dose-dumping effect has important implications for label claims for dose administration.

3.1.1. Solubility and Permeability

As stated above, the two most important physico-chemical determinants for drug absorption are solubility and permeability. Lipinski et al. (6,8)

suggested that compounds with aqueous solubility less than 5 $\mu\text{g/mL}$ (10 μM) and polar surface area (relates to permeability for compounds that are passively absorbed) greater than 150–200 μ^2 rarely achieve good absorption and bioavailability. It has been estimated that roughly 85% of marketed drugs are highly soluble ($>65 \mu\text{g/mL}$). Poor solubility can sometimes be overcome by developing an appropriate formulation to increase solubility (6,8). However, some formulations may also enhance permeability due to their effects on membrane transporters (see below). Based on the solubility and permeability properties, drugs are classified into one of the following six categories, known as the Biopharmaceutics Classification System (BCS) (29–32):

- Class 1: high solubility and high permeability,
- Class 2: low solubility and high permeability,
- Class 3: high solubility and low permeability,
- Class 4: low solubility and low permeability,
- Class 5: high solubility and intermediate permeability, and
- Class 6: low solubility and intermediate permeability.

The classification of a drug to the appropriate BCS class early in drug discovery is becoming an important step in predicting its pharmacokinetic behavior in animals and humans. This aids in the estimation of the required resources and the length of the time for the preclinical and clinical development of a compound.

The BCS was implemented by the FDA to waive bioequivalence studies for generic drugs with Class 1 properties (29). Many drugs in this class were formulated for extended release (33). Class 1 compounds include hydrophilic drugs that are not ionized in the GI tract (e.g., acetaminophen, valproic acid, ketoprofen, disopyramide, verapamil, and metoprolol) (10). For a compound with good membrane permeability, dissolution is generally the rate-limiting step. Here, attempts are made to establish *in vitro*–*in vivo* correlations between dissolution testing in the laboratory and clinical observations (i.e., drug absorption in man). Ideally, it is highly desirable to develop new drug candidates that have Class 1 properties. However, in reality it is not practical, as many Class 1 compounds may not have good efficacy due to their poor lipophilicity.

The absorption and oral bioavailability of Class 2, Class 3, and Class 4 compounds may depend on several factors and, therefore, these compounds may not have desirable pharmacokinetic properties. They may require specific administration conditions including novel formulations, fasted/fed state, among many others, to improve their oral absorption (13,15). The ionization state of the drug, the physico-chemical interactions of the drug with specific components in the formulation or meal, can influence the pH of the GI tract, dissolution and diffusion rate resulting in either increased or decreased absorption of drug.

For example, a meal high in fat content increases the solubilization of nonionizable poorly soluble Class 2 drugs and thereby increases the absorption. However, for weakly basic Class 2 drugs, the high fat meal may have diverse effects depending on intrinsic solubility, intestinal pH, pK_a , and caloric density of the meal. For a drug with narrow therapeutic margin, the increase in absorption may actually result in undesirable adverse events rather than providing the desirable pharmacokinetic properties.

Certain formulations and meals can influence the GI pH. For weakly basic drugs with low pK_a , the elevated pH in the GI tract will cause precipitation of the drug, resulting in poor absorption. The concentration of the drug that is in dissolved state in the intestinal lumen may be influenced by luminal pH. At higher pH, weakly acidic drugs (e.g., NSAIDs) dissolve faster than the weakly basic drugs (e.g., Indinavir, HIV protease inhibitor).

Class 3 compounds are relatively impermeable, but have high aqueous solubility. Permeability is measured using several *in vitro* models, including Caco-2 cells (34), MDCK cells (35), PAMPA (10) *in situ* intestinal permeability models (36–38) and excised intestinal segments (39). It is generally believed that there is a direct relation between permeability coefficient and the extent of absorption of compounds. Although in some cases, it has been possible to directly correlate absorption with permeability, more often a very poor correlation observed. The common factor among the poorly permeable but well-absorbed drugs appears to be their high aqueous solubility, generally exceeding 2.5 mg/mL. Hilgers et al. (40) recently studied a series of antibacterial oxazolidinones, which have strikingly different absorption profiles and poor permeability coefficients. High aqueous solubility appears to help compensate for the poor *in vitro* permeability observed.

Many compounds in Class 3 also show differences in absorption across the GI tract, with better absorption in the upper small intestine (13). Therefore, any interactions in the upper GI tract may result in a significant decrease in oral bioavailability. For example, administration of Class 3 medications with food can decrease the absorption of these compounds, independent of fat content in the meal.

Class 4 drugs are both poorly soluble and poorly permeable. Poor aqueous solubility does not necessarily mean high lipophilicity. Thus, a high fat meal may not necessarily enhance the absorption of these drugs. These drugs generally display very low oral bioavailability. The decision to develop such compounds depends on their high therapeutic potency and safety margin (13). If a drug of this class has therapeutic potential for a specific disease of the GI tract, then low solubility and low permeability are minor drug development issues. An example of medications in this category are nonabsorbable antibacterials (e.g., colistin, tobramycin sulfate, and amphotericin B). These agents are used for selective decontamination of the GI tract prior to surgery and to prevent the development of particular disease states.

3.1.2. Factors Influencing Solubility

Although the BCS classification is very useful during drug discovery in predicting drug absorption and bioavailability, several other physicochemical factors including crystalline form, particle size, and ionization state may influence these predictions (41).

The aqueous solubility of highly crystalline drugs varies with crystal form, and for the most stable crystal form, the aqueous solubility is generally low. The formation of high energy, low-melting crystal or amorphous solids frequently yields more rapidly dissolving and higher solubility forms of the drug. Amorphous forms are generally used during drug discovery, and therefore the solubility determinations using these forms may result in an erroneous BCS classification for the compound. Similarly, early pharmacokinetic studies conducted in drug discovery may result in high bioavailability estimates, which later may prove to be much lower when a highly crystalline form of the compound is used during drug development (e.g., PNU-103017) (42). Therefore, the determination of melting points and energy states could also be an important factor during screening in drug discovery.

Another factor that influences the solubility of drug is its particle size. In general, reducing the particle sizes can enhance the solubility and, consequently, enhance absorption. For example, danazol is a poorly water-soluble drug (10 $\mu\text{g/mL}$) and shows poor bioavailability ($\sim 5\%$) in humans and dogs. Liversidge and Cundy (43) showed that reducing the particle size of danazol to an average of 85 nm (nanoparticle dispersion) increased bioavailability to 82% in dogs. Interestingly, particle size not only influences the solubility of drug but also the receptor/enzyme-binding interactions.

The solubility and absorption properties of ionizable drugs can depend upon the pH characteristics of the GI tract and may result in significant pharmacokinetic variability among the human population. For example, cinnarazine is a very insoluble drug (15 ng/mL) with two basic groups (pK_a values of 1.94 and 7.47, respectively). This drug is very soluble in acidic solutions and its absorption is dependent on the gastric pH (41,44). In individuals with high gastric acid content (i.e., low gastric pH), cinnarazine has good absorption characteristics. Conversely, in those individuals showing low-gastric acid content, AUC and C_{max} were reduced by approximately 75–85%.

3.2. Carrier-Mediated Transport

Another route for intestinal transport involves active transport and secretory processes in the epithelial cells for which many common drugs are substrates for these transport proteins (45,46). It has been estimated that about 2% of human genome encode membrane transporters and related proteins, suggesting a potentially important role in transporting endogenous and exogenous small molecules, peptides, and proteins. Transporters have been

classified as primary, secondary, and tertiary active transporters. The primary transporters include ATP-binding cassette transporters such as MDR, MRP, and BCRP, which are dependent on ATP-hydrolysis. The secondary and tertiary active transporters include OAT, OATP, NTCP, OCT, OCTN, and PEPT, which are dependent on exchange or cotransport of intracellular and/or extracellular ions. Recently, the Human Genome Nomenclature Committee has classified transporters using standardized names, such as the solute carrier superfamily (SLC) and ATP-binding cassette (ABC) transporters (Table 2). The role of membrane transporters on drug disposition is the focus of [Chapter 8](#), where the reader will find more detailed information.

Table 2 Human Drug Transporters: Standardized Classification According to Human Gene Nomenclature Committee (HGNC)

Name	HGNC symbol	Conventional symbol(s)
Multidrug resistant gene/P-glycoprotein	<i>ABCB1</i>	MDR1-P-gp
Bile salt export pump/sister P-glycoprotein	<i>ABCB11</i>	BSEP/SPGP
Multidrug resistance associated protein 1	<i>ABCC1</i>	MRP1
Multidrug resistance associated protein 2	<i>ABCC2</i>	MRP2/cMOAT
Multidrug resistance associated protein 3	<i>ABCC3</i>	MRP3
Multidrug resistance associated protein 4	<i>ABCC4</i>	MRP4
Breast cancer resistance protein	<i>ABCG2</i>	BCRP
Sodium taurocholate cotransporting peptide	<i>SLC10A1</i>	NTCP
Apical sodium-dependent bile acid transporter	<i>SLC10A2</i>	ASBT
Oligopeptide transporter 1	<i>SLC15A1</i>	PEPT1
Oligopeptide transporter 2	<i>SLC15A2</i>	PEPT2
Organic anion transporting polypeptide-A	<i>SLC21A3</i>	OATP-A
Organic anion transporting polypeptide-C	<i>SLC21A6</i>	OATP-C/OATP2/ LST-1
Organic anion transporting polypeptide-8	<i>SLC21A8</i>	OATP-8
Organic anion transporting polypeptide-B	<i>SLC21A9</i>	OATP-B
Organic anion transporting polypeptide-D	<i>SLC21A11</i>	OATP-D
Organic anion transporting polypeptide-E	<i>SLC21A12</i>	OATP-E
Organic anion transporting polypeptide-F	<i>SLC21A14</i>	OATP-F
Organic cation transporter 1	<i>SLC22A1</i>	OCT1
Organic cation transporter 2	<i>SLC22A2</i>	OCT2
Organic cation transporter 3	<i>SLC22A3</i>	OCT3
Novel organic cation transporter 1	<i>SLC22A4</i>	OCTN1
Novel organic cation transporter 2	<i>SLC22A5</i>	OCTN2
Organic anion transporter 1	<i>SLC22A6</i>	OAT1
Organic anion transporter 2	<i>SLC22A7</i>	OAT2
Organic anion transporter 2	<i>SLC22A8</i>	OAT3
Organic anion transporter 4	<i>SLC22A9</i>	OAT4

Transporters involved in drug absorption include influx transporters (PEPT1, ASBT, OATP-B, OATP-D, OATP-E) and efflux transporters (Pgp, MRP2, or BCRP) that are expressed in the gut. Understanding the involvement of these transporters early in drug discovery can help in predicting the clinical outcome and planning an appropriate strategy for the development of the drug.

Of all the drug transporters expressed in GI tract, the most widely studied is Pgp, and a number of drugs are substrates to this efflux transporter (46). Pgp-dependent efflux can result in poor absorption and bioavailability of medications. Therefore, inhibition of Pgp is expected to increase the absorption and bioavailability of drug substrates of this transporter. For example, it has been shown that TPGS (d- α -tocopheryl polyethylene glycol 1000 succinate), a water-soluble vitamin E analog, enhanced the absorption of cyclosporine (a Pgp substrate) in healthy volunteers and liver transplant patients, which is attributed to inhibition of Pgp by TPGS (47). TPGS also increases the solubility of amprenavir, a HIV protease inhibitor as well as inhibited Pgp resulting in enhanced drug permeability through Caco-2 cell monolayers (48). Surfactants such as Cremophor EL/RH40 or Tween 80 have also been found to be potent inhibitors of Pgp, and formulations containing these agents have been used as vehicles for poorly water-soluble anticancer drugs such as paclitaxel and docetaxel (48–51). Cremophor RH40 has been shown to enhance the absorption of digoxin in human volunteers, approximately by 20%, supporting the *in vitro* findings (50). Tween 80 was shown to increase the apical-to-basolateral permeability of furosemide, cimetidine, and hydrochlorothiazide in Caco-2 cells, presumably by inhibiting their active efflux by Pgp (46). Similarly, Pluronic P85 increased the absorptive permeability of fluorescein, doxorubicin, paclitaxel, etoposide, and azidodeoxythymidine through Caco-2 cells (52).

Arima et al. (53) demonstrated that 2,6-di-*O*-methyl- β -cyclodextrin not only increased the solubility and dissolution rate of the immunosuppressive agent tacrolimus but also inhibited Pgp. Therefore, agents that increase the solubility and inhibit the efflux transporters in the intestine can enhance oral bioavailability, particularly in cases where drug absorption is limited by both solubility and permeability (53,54). In addition, drug–drug interactions of Pgp substrates and/or inhibitors can result in increased oral bioavailability of some drugs (45,46). For drugs belonging BCS Class III and IV (poorly permeable compounds), these factors should be evaluated early enough in preclinical development, so the information generated can be helpful in making critical go/no go decisions before futile investments are made.

Interestingly, food components and herbal remedies also seem to inhibit the Pgp and increase drug absorption (51,55,56). However, in some cases, these compounds (e.g., St. John's Wort) also downregulate or upregulate Pgp expression (57) as well as cytochrome P450 enzymes (58,59). [Table 3](#) shows some known interactions of Pgp substrates with food components.

Table 3 Effects of Food and Herbal Products on P-glycoprotein (Pgp) Transport

Food type	Effect on Pgp	Study type	Affected compound(s)
Green tea	Inhibition	In vitro	Rhodamine-123, vinblastine
Rosemary extract	Inhibition	In vitro	Doxorubicin, vinblastine
St. John's Wort	Induction	In vitro	Rhodamine-123
	Stimulation	In vitro	Vinblastine, digoxin, cyclosporin A
Grapefruit juice	Inhibition	In vivo	Cyclosporin A, talinolol, dextramethorphan
	Inhibition	In vitro	Vinblastine, rhodamine-123, saquinavir
Seville orange juice	Inhibition	In vitro	Vincristine, vinblastine
	Inhibition	In vivo	Dextromethorphan
Orange juice	Inhibition	In vitro	Rhodamine-123, saquinavir
Orange extract (standardized)	Inhibition	In vitro	Cyclosporin A
Strawberry extract (standardized)	Inhibition	In vitro	Cyclosporin A
Dietary fatty acids	Inhibition	In vitro	Digoxin
Piperine (black pepper)	Inhibition	In vitro	Digoxin, cyclosporin A
Peppermint oil	Inhibition?	In vivo	Cyclosporin A ^a
Mint extract (standardized)	Inhibition	In vitro	Cyclosporin A
Apricot extract (standardized)	Inhibition	In vitro, ex vivo, in situ	Talinolol

^aIncreased absorption of cyclosporin A, apparently not due to inhibition of metabolism.

3.3. Bioavailability Determinations

One of the important first stages in drug development involves assessment of the absolute bioavailability of a new compound (12). Bioavailability is defined as the fraction of administered dose reaching the systemic circulation. Even if a drug is completely absorbed, bioavailability may be low due to presystemic metabolism or degradation in the GI tract.

Typically bioavailability is determined by dosing the drug using two routes. The test product is the intended clinical route of administration (oral, dermal, intra-peritoneal, intra-muscular, intranasal, etc.). Bioavailability is calculated as the ratio of dose-normalized AUC (test vs. reference products). For absolute bioavailability, the reference product is an intravenous dose. If absolute bioavailability is less than 5%, then the drug is considered to be poorly bioavailable. However, this may not be an issue for a highly potent compound, since this bioavailability may be sufficient to produce the desired clinical response. Therefore, one may argue that

bioavailability is not a very important factor, particularly when safe plasma concentrations well above the target efficacious concentrations are achievable by the intended clinical route. Nevertheless, a comparison of absolute bioavailabilities in different animal species could provide important information with regard to permeability, absorption, and drug metabolism across species.

Poor bioavailability may sometimes also be the result of extensive hepatic clearance and/or hepatic metabolism rather than poor absorption. In such cases, permeability data in Caco-2 cells and in vitro metabolism studies in liver microsomes/hepatocytes could help guide the preclinical programs.

3.3.1. Formulation Selection for Preclinical Toxicology Studies

Often, bioavailability studies in animals are very useful for appropriate design of preclinical toxicology studies. For a poorly bioavailable drug, even when the compound is highly potent, it is important to achieve sufficient plasma concentrations and exposure in order to achieve measurable toxicity and to calculate the margin of safety. Early preclinical toxicology studies are generally conducted with escalating doses usually up to MTD, in some cases as high as 2000 mg/kg. Unfortunately, in most cases, drugs are insoluble at those high doses and, therefore, are poorly absorbed. As a result, a dose-response relationship up to MTD may not be achieved. Therefore, it is critical to use a vehicle that solubilizes the drug at high doses, before toxicology studies are conducted. It is now routine practice in early drug development to use common vehicles that solubilize the drug to desired concentrations. Several formulation additives (e.g., PEG-400, hydroxypropyl β -cyclodextran, Tween, propylene glycol, cremophore, and VitaminE-TPGS) are known to enhance the solubility of compounds (60,61). These agents can be utilized to enhance drug absorption, resulting in a dose-dependent increase in drug exposure and toxicity. In addition, some of these formulations also increase drug absorption by inhibiting Pgp-dependent efflux, as described previously in this chapter.

4. MECHANISMS OF SMALL MOLECULE DISTRIBUTION

Distribution is one of the two critical determinants of drug disposition, the other being clearance. Once a compound reaches the systemic circulation, it is available for distribution throughout the body. While the therapeutic effect of the drug will depend on its ability to access its site of action or “biophase,” drug distribution to other organs and tissues can result in adverse or toxic effects. Additionally, sequestration of drug in an organ or tissue may result in a prolonged residence time in the body (i.e., a long elimination half-life).

The extent of drug distribution in the body is described by V_D . V_D is defined as proportionality constant between the amount of drug in the body and its concentration in the plasma. In preclinical development, it is important to characterize the distribution pattern of a new chemical entity. This is typically done in small animal models as part of a toxicokinetic assessment. As discussed in [Chapter 10](#), tissue distribution studies are conducted in accordance with ICH guidelines. Through these studies, information is obtained regarding the distribution and accumulation of drug and metabolites, particularly in relation to potential sites of action (62).

Since a goal of clinical pharmacokinetics is to establish and target useful therapeutic ranges of drugs, a relationship between plasma concentrations and those at the “biophase” is assumed to exist. The concept that relates these concentrations is distributional equilibrium (DE). DE occurs when the unbound concentration in the plasma is equal to unbound concentration of drug in the tissue. Two important characteristics of distribution are rate and extent. In other words, “how quickly does a drug distribute?” and “where is the drug going in the body?” To answer the first question, the rate of distribution depends on two factors: blood flow and the ability of the drug to cross biological membranes. The rate-limiting step to distribution is blood flow to the organ or tissue. Organs such as the liver, kidney, heart, and brain are *highly perfused* and drug reaches these organs rapidly. On the other hand, adipose tissue and skeletal muscle are *poorly perfused*, and it takes time for drug to reach them.

In addition to blood flow, the ability of drug to penetrate a biological membrane also affects the rate of distribution. There are two general types of distribution mechanisms: passive uptake, and carrier-mediated transport. When drug uptake into organs and tissues is a passive process, it can be described by Fick’s Law of Diffusion. Therefore, the rate of absorption depends upon the drug’s lipophilicity (partition coefficient), the thickness of the absorbing membrane, ionization state (pH vs. pK_a), and plasma protein binding. Ionization is important because of the pH partition hypothesis that assumes that a drug molecule can be absorbed only in its unionized form. Since the driving force for passive diffusion is the concentration gradient across the membrane, if the drug is highly and strongly bound to plasma protein this concentration gradient will be reduced.

Besides the rate of distribution (i.e., time required to reach DE), it is also important to consider the extent of distribution. Here, protein binding is most important, although lipophilicity/polarity and ionization are critical determinants as well. Most drugs can readily exit capillaries. Albumin, the primary binding protein in the plasma, is a large molecule (average molecule weight 69,000 Da) which is unable to cross the capillary wall and leave the bloodstream. Therefore, any protein-bound drug cannot leave the plasma. This is particularly important for weakly acidic compounds (e.g., furosemide, phenytoin). Generally speaking, the volume of distribution of weak

acids is relatively small (<0.5 L/kg). This is not only due to plasma protein binding, but also because of poor lipophilicity. For example, aminoglycosides do not bind to plasma albumin and are therefore free to exit the capillary and access the extracellular fluid (ECF). However, these compounds do not cross biological membranes very well and do not distribute further. The reported V of these medications is 0.25 L/kg (63).

In contrast to weak acids, weakly basic drugs are generally lipophilic and not highly plasma protein bound. Because of their physicochemical characteristics, lipophilic compounds readily cross biological membranes and are taken up by tissues. In many cases, the tissue can act as a reservoir for drug, and drug is assumed to be “bound-up” by the tissue. An example is the tricyclic antidepressants. The volume of distribution of these compounds can approach 60 L/kg (64), indicating that they do not stay in the bloodstream, but rather are sequestered somewhere else in the body. Drugs with large volumes usually take time to distribute once they are administered, and distribution is extensive.

Besides passive uptake, carrier-mediated transport contributes to drug distribution. In addition to the intestine (discussed above), numerous transporters are expressed in other tissues, including the liver, kidney, and brain. Recent evidence has begun to elucidate the role of membrane transporters in the placenta, mammary gland, and testes. Therefore, transporters provide an important mechanism for the distribution of small molecules.

For example, in the development of therapeutic agents that require requiring CNS penetration for their efficacy (e.g., HIV medications, chemotherapeutic agents), membrane transporters significantly impact drug efficacy (65). Likewise, adverse effects of medications can be reduced through an understanding of the role of membrane transports of CNS distribution. For more details on the role of membrane transporters in the CNS, the reader is referred to [Chapter 8](#).

Based upon the previous discussion, it is evident that plasma protein binding can affect both the rate and extent of drug distribution. The primary binding protein in plasma is albumin, which binds primarily weakly acid molecules (salicylic acid, phenytoin). The large size of this molecule prevents it (and anything bound to it) from exiting the capillary. Therefore, drugs that are highly bound to albumin are generally restricted to the bloodstream. A second important binding protein is alpha-1 acid glycoprotein (AAG) which binds some weak bases such as propranolol and quinidine. AAG is not the primary binding protein, but the interesting fact about this molecule is that AAG levels can increase or decrease secondary to disease and other factors. Stress, surgery, and malignancy all increase AAG while pregnancy, malnutrition, and liver disease can decrease AAG. Perturbations in AAG can affect drug binding, distribution, and, in certain instances, therapeutic activity (66). Likewise, plasma lipoproteins are potential binding targets for hydrophobic compounds (e.g., cyclosporine A).

Changes in the lipoprotein plasma profile of an individual can potentially influence drug disposition and drug activity (67).

Like AAG, changes in albumin plasma concentrations can also affect drug distribution and response. Hypoalbuminemia can occur in elderly patients and those with renal failure. A decrease in the concentration of plasma albumin will increase drug free fraction (f_u) which increases V . Phenytoin is a useful example (68). Patients with hypoalbuminemia will have an increased f_u of phenytoin which can potentially result in toxicity (renal failure will further complicate this as uremia increases f_u of phenytoin even more).

A recent review by Benet and Hoener (69) demonstrated that protein binding changes caused by drug–drug and disease drug interactions are rarely of clinical importance. Except in rare cases (e.g., a compound with a high extraction ratio and narrow therapeutic range), an increase in f_u will result in increased drug clearance. Consequently, clinical exposure of a patient to the drug will be unaffected. Nevertheless, protein binding measurements are important during drug development for several reasons. First, interspecies differences in f_u will affect allometric predictions of pharmacokinetic parameters (clearance, volume of distribution). Second, knowledge of drug protein binding (f_u) is necessary for establishing a suitable first dose in humans. Third, therapeutic drug monitoring typically involves measuring total drug concentrations. For a highly protein-bound compound with a narrow therapeutic range (e.g., phenytoin), this can result in erroneous dosing adjustments for patients with elevated f_u . In the phenytoin example described above, patients with reduced protein binding of phenytoin (e.g., secondary to hypoalbuminemia) tend to have lower total drug concentrations (i.e., below the established therapeutic range), which are often misinterpreted. In this case, attempts should be made to extrapolate observed drug concentrations to “normal binding” conditions in order to avoid unnecessary increases in dose (69).

In addition to plasma proteins, other components of the blood may influence drug disposition. Specifically, the erythrocytes are a potentially important distribution site for medications. The erythrocytes play an important role in the transport and disposition kinetics of medications (e.g., carbonic anhydrase inhibitors) in the blood (70,71). However, the erythrocytes are often regarded as an insignificant compartment of drug distribution. For those compounds that accumulate in the erythrocytes to an appreciable extent, characterization of different kinetic events occurring within the erythrocyte can provide significant insight into drug disposition (72).

5. MECHANISMS OF SMALL MOLECULE METABOLISM

Clearance is the most important determinant of drug disposition, and it is the parameter used to establish suitable doses of medications. Drug

metabolism plays a major influential role in inter-individual variability in drug clearance in humans. Drug metabolism may be altered in disease states including hepatic and renal failure, resulting in variable drug clearance in humans. Other factors contributing to variability include pharmacogenetics (e.g., polymorphism), gender, age, and drug interactions.

Drug is cleared from the body through two general pathways: metabolism and excretion. As discussed below, excretion implies the elimination of drug from the body intact. While excretion is the predominant pathway for some compounds, many medications undergo some degree of metabolism or biotransformation *in vivo*. Drug metabolism involves chemical modification of a compound in the body. The resulting metabolites that are formed (either active or inactive) either undergo further metabolism or are excreted by the body. Drug metabolism is an important mechanism for the elimination of lipophilic molecules. These compounds require biotransformation to more polar metabolites that can be readily excreted. Presented here is an overview of drug metabolism. For further information, a number of textbooks are available (73,74).

The liver and intestine are considered the major sites of drug metabolism. There are two general types of metabolic reactions: phase 1 and phase 2. Phase 1 metabolism includes reactions involved in the biotransformation of molecules that lack a required functional group for conjugation reactions (phase 2). Some of the important enzymes that catalyze phase 1 drug metabolism in these organs are cytochrome p450 (CYP), flavin monooxygenases (FMO), xanthine oxidase, and aldehyde oxidase. Phase 2 enzymes include glucuronosyl transferases (GT), *n*-acetyltransferase, and sulfotransferases (ST). Several of these enzyme systems are discussed below.

5.1. Phase 1 Enzymes

5.1.1. Cytochrome P-450 (CYP)

CYP is the primary enzyme system responsible for the oxidative metabolism of xenobiotics. CYP enzymes are primarily located in the endoplasmic reticulum of cells, when fractionated form vesicles called microsomes. The name P450 stems from absorbance wavelength of the activated enzyme (450 nm), when carbon monoxide was added to reduced microsomes with NADH or dithionite. CYP is a family of enzymes that are classified based upon the structural similarity of their amino acid sequence (73). Enzymes having >40% sequence identity belong to the same family (e.g., CYP1, CYP2). Those enzymes with >55% overlap are grouped into subfamilies (e.g., CYP1A). Eukaryotic enzymes (those identified in eukaryotic systems) are designated CYP100 or less. A list of CYP isoforms across various species is provided in [Table 4](#).

The most abundant (>30% total content) CYP enzyme in the human liver is CYP3A4, one of the two primary enzymes for drug metabolism. The

other enzyme, CYP2D6, makes up <2% of total CYP. The predominant isoforms of interest include CYP1A2, CYP2A6, CYP2C8/9/10/19, CYP2D6, CYP2E1, and CYP3A4. Of these CYP3A4, CYP2D6, and CYP2C9 are involved in the metabolism of about 50%, 15%, and 15% of the known drugs, respectively. In addition, many of the CYPs are polymorphic whose expression in human liver is genetically controlled (see below).

A number of reactions are catalyzed by CYP (73,74). Examples include oxidative and reductive mechanisms. The oxidative reactions include aromatic and side-chain hydroxylations, *N*- and *O*-dealkylations, deamination of primary and secondary amines, *N*-oxidations, sulfoxidations, desulfurations, and ester cleavage. Reductive reactions catalyzed by CYP include reduction of epoxides, *N*-oxides, nitroso compounds, hydroxylamines, nitro compounds, azo compounds, nitrosamines and azido compounds, and reductive dehalogenation. The reduction reactions of *N*-oxides, nitroso compounds, and hydroxylamines are the counterpart of oxidative reactions catalyzed by CYP and should be viewed as bioreversible oxidation–reduction reactions. In contrast, the reduction of nitro, azo azido compounds, and nitrosamines are not mirrored by their oxidative formation and, therefore, are not bioreversible oxidation–reduction reactions.

There is a high degree of intersubject variability in drug metabolism. Genetic differences in metabolism are reflected in enzyme polymorphism. All the major human CYP enzymes responsible for drug metabolism exhibit common polymorphisms at genomic level (75). Human CYP3A gene encodes at least four isoforms including CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A4 and CYP3A5 are found in adult liver, intestine, and kidney. They have similar substrate specificity but not identical. CYP3A5 is predominantly expressed in 60% African Americans and in about 33% Caucasians. When CYP3A5 is expressed in adult liver, the level is usually about 50% of total CYP3A content in the liver. CYP3A7 is found in intestine, reproductive organs, and infant liver but is also expressed in some adult livers. CYP3A43 gene is predominantly found in prostate and in many other tissues including liver; however, this isoform appears to be a nonfunctional CYP3A isoform.

Both CYP3A4 and CYP3A5 are polymorphic (75,76). For CYP3A4, a total of 28 SNPs have been identified, five of which produced coding changes: CYP3A4*3, *15, *17, *18, *19. Interestingly, all these polymorphisms of CYP3A4 gene produce active CYP3A4 without inactivating the gene or the protein. Racial differences were found in these polymorphisms: CYP3A*15 was found only in blacks, with 4% allelic frequency, CYP3A*3 and *17 were found in Caucasians with allelic frequency of 2% and 4%; CYP3A*18 and *19 were identified in Asians with allelic frequency of 2%. Although, CYP3A4 polymorphisms do not result in loss of activity, they catalyze the metabolism of their substrates at different turnover rates. Some of these polymorphisms also appear to affect the inducibility of CYP3A4.

Table 4 Cytochrome P450 Isoforms: A Species Comparison

CYP family	Human	Mouse	Rat	Rabbit	Dog	Monkey
1A	1A1, 1A2	1a1, 1a2	1A1, 1A2	1A1, 1A2	1A2	1A1
1B	1B1	1b1	1B1	—	—	—
2A	2A6, 2A6v2, 2A7, 2A13, 2A18PC, 2A18PN	2a4, 2a12	2A1, 2A2, 2A3	2A10, 2A11	—	—
2B	2B6, 2B7P	2b9, 2b10, 2b13, 2b19, 2b20, 2b20P1	2B1, 2B2, 2B3, 2B8, 2B12, 2B14P, 2B15, 2B16P, 2B21	2B4, 2B5	2B11	2B17
2C	2C8, 2C9, 2C18, 2C19	2c29, 2c29v2, 2c37, 2c38, 2c39, 2c40, 2c50, 2c51, 2c52P, 2c53P, 2c54, 2c55	2C6, 2C7, 2C11, 2C12, 2C13, 2C22, 2C23, 2C24	2C1, 2C2, 2C3, 2C4, 2C5, 2C14, 2C15, 2C30	2C21, 2C41	2C20, 2C43
2D	2D6, 2D7AP, 8BP	2d10, 2d11, 2d12, 2d13, 2d22, 2d26	2D1, 2D2, 2D3, 2D4, 2D5, 2D18	2D23, 2D24	2D15	2D17, 2D29
2E	2E1	2e1	2E1	2E2	2E1v1, 2E1v2	2E1
2F	2F1P	2f2	2F4	—	—	—
2J	2J2	2j5, 2j6, 2j7, 2j8, 2j9	2J3, 2J3P1, 2J3P2, 2J4	2J1	—	—
2R	2R1	—	—	—	—	—
2S	2S1	2s2	—	—	—	—
2T	2T2P, 2T3P	?	2T1	—	—	—
2U	2U1	—	—	—	—	—
2W	2W1	—	—	—	—	—
3A	3A3, 3A4, 3A5,	3a11, 3a13, 3a16,	3A1, 3A2, 3A9,	3A6	3A12, 3A26	3A8

	3A5P1, 3A5P2, 3A7, 3A43	3a25, 3a41, 3a44	3A18, 3A23			
4A	4A11	4a10, 4a12, 4a14, 4a22	4A1, 4A2, 4A3, 4A8	—	4A4, 4A5, 4A6, 4A7	—
4B	4B1	4b1	4B1	4B1	—	—
4F	4F2, 4f3, 4F3v2, 4F8, 4F9P, 4F10P, 4F11, 4F12, 4F22, 4F23P, 4F24P, 4F25P, 4F26P, 4F27P	4f13, 4f14, 4f15, 4f16, 4f17, 4f18	4F1, 4F4, 4F5, 4F6, 4F9	—	—	—
4V	4V2	4v3	—	—	—	—
4X	4X1	4x1	4X1	—	—	—
4Z	4Z1	—	—	—	—	—
5A	5A1	5a1	5A1	—	—	—
7A	7A1	7a1	7A1	7A1	—	—
7B	7B1	7b1	7B1	—	—	—
8A	8A1	8a1	8A1	—	—	—
8B	8B1	8b1	—	8B1	—	—
11A	11A1	11a1	?	11A1	—	—
11B	11B1, 11B2	11b1, 11b2	11B1, 11B2, 11B3, 11B8P	—	—	—
17	17	?	17	—	—	—
19	19	19	19	—	—	—
20	20	20	—	—	—	—
21	21A1P, 21A2	—	21	—	—	—
24	24	—	24	—	—	—

(Continued)

Table 4 Cytochrome P450 Isoforms: A Species Comparison (*Continued*)

CYP family	Human	Mouse	Rat	Rabbit	Dog	Monkey
26A	26A1	26a1	—	—	—	—
26B	26B1	—	—	—	—	—
26C	26C1	26c1	—	—	—	—
27A	27A1	—	27A1	27A1	—	—
27B	27B1	27B1	27B1	—	—	—
27C	27C1	—	—	—	—	—
39	39	39	—	—	—	—
46	46	—	—	—	—	—
51	51, 51P1, 51P2	—	51	—	—	—

Polymorphisms of CYP3A5 were also described: CYP3A5*1 allele expresses the active CYP3A5 protein while CYP3A5*3 and CYP3A5*6 alleles produce no CYP3A5 protein.

CYP2D6 polymorphisms are of major concern as many of its substrates have narrow therapeutic margin (77). More than 50 alleles of CYP2D6 have been described in literature. There are three categories of individuals (poor metabolizers, extensive metabolizers, and ultra-rapid metabolizers) depending on the nature of CYP2D6 polymorphisms. Poor metabolizers have inactivating mutations resulting in inactive or no protein expression. Ultra-rapid metabolizers possess several copies of CYP2D6 producing excessive active protein. Approximately 7% of the Caucasian population and <1% of Orientals and African-Americans are poor metabolizers for CYP2D6. This can lead to problems with certain classes of medications (e.g., antidepressants, antiarrhythmics) due to increased plasma levels of drug in these patients. For example, poor CYP2D6 metabolizers respond poorly to codeine therapy because the analgesic activity of codeine depends upon its conversion to morphine in vivo via CYP2D6 (78). In contrast for oxycodone CYP2D6 polymorphisms had little consequence on PK/PD of oxycodone in poor metabolizers, due to a compensatory increase in metabolism via CYP3A4. The poor metabolizer phenotype of CYP2C19 is present in 20% of Asians, but only 3% in Caucasians (79). An example here is omeprazole. Omeprazole induces CYP1A2, but is a substrate for CYP2C19. In CYP2C19 poor metabolizers, omeprazole may be a more potent inducer of CYP1A2 because of elevated plasma levels of drug (80).

The dramatic variability in enzyme expression is a factor contributing to drug-related problems in patient care, a result of administration of an insufficient dose to an ultra-metabolizer or a toxic dose to a slow metabolizer. Enzyme phenotyping involves administration of a probe drug for a specific enzyme and comparing the urinary recovery of the probe and its metabolite. A slow metabolizer would be expected to have a low urinary metabolite ratio (metabolite:parent in the urine), where a rapid metabolizer would have a high urinary metabolite ratio. Table 5 lists probe substrates for phenotyping various P450 enzymes. Presently, phenotyping is limited by the time and resources required for testing. As a result, widespread utilization of patient phenotyping is presently impossible. However, cocktail approaches with simultaneous administration of several CYP substrates appear to be a viable alternative approach for CYP phenotyping (81,82). This approach was successfully applied for assessing drug-drug interactions in the clinical setting.

5.1.2. Other Phase 1 Enzyme

It should be noted that some metabolic reactions are not exclusively catalyzed by CYP. For example, *N*-oxidations, *S*-oxidations are also catalyzed flavin monooxygenases which are present in the liver and require NADPH

Table 5 CYP Phenotyping: Examples of CYP Probe Compounds

CYP isoform	Probe compound(s)
CYP1A2	Theophylline, caffeine
CYP2B6	Bupropion
CYP2C9	<i>S</i> -Warfarin, tolbutamide
CYP2C19	<i>S</i> -Mephenytoin, omeprazole
CYP2D6	Desipramine, debrisoquine, dextromethorphan
CYP2E1	Chlorzoxazone
CYP3A	Midazolam, busprione, felodipine, simvastatin, lovastatin

to catalyze these reactions. Similarly, *N*-dealkylations and oxidations of phenols can be catalyzed by other hemeproteins including myeloperoxidase, eosinophil peroxidase, prostaglandin H synthase, etc., although the mechanisms of catalysis are different from CYP-catalyzed reactions. In addition, metabolic products of hydrolytic esterase reactions are similar to oxidative ester cleavage although the underlying mechanisms of catalysis are quite different. In addition, oxidation of azaheterocycles is catalyzed by molybdenum hydroxylases, aldehyde, and xanthine oxidases.

5.2. Phase 2 Enzymes

Phase 2 reactions are conjugation reactions. Conjugating agents are the byproduct of protein, carbohydrate, and fatty acid metabolism. A list of phase 2 pathways is provided in Table 6.

Compared to phase 1 metabolism, phase 2 reactions are faster, but they have a limited capacity. In other words, these are more readily saturable. This is the important factor involved in metabolism-based toxicity and carcinogenesis. Generation of toxic species (through phase 1 metabolism) may overwhelm phase 2 detoxification pathways, resulting in cell death. Reasons for limited phase 2 capacity include the following: lim-

Table 6 Phase 2 Reactions

Reaction	Conjugating agent	Reactive intermediate	Functional groups
Glucuronidation	Glucuronic acid	UDPGA ^a	–OH, –NH ₂ –COOH, –SH
Acetylation	Acetyl CoA	AcetylCoA	OH, –NH ₂
Sulfate conjugation	Sulfate	PAPS ^b	OH, –NH ₂
Mercaptopuric acid synthesis	Glutathione	Arene oxides, epoxides	Arene oxides, epoxides

^aUDPGA, uridine diphosphate glucuronic acid.

^bPAPS, 3'-phosphoadenosine-5'-phosphosulfate.

ited amount of available enzyme (transferase), limited ability to synthesize active intermediate, and limited amount of conjugating agent. An example of the latter is glutathione depletion during acetaminophen overdose (described below). However, phase 2 conjugation is not necessarily a detoxification pathway. For example, acyl glucuronides can covalently bind to tissue proteins and result in toxicity.

5.2.1. Glucuronidation

Glucuronidation represents the major phase 2 conjugation pathway in drug metabolism reactions (83–85). Several functional groups are substrates for glucuronidation reactions, which include *O*-glucuronidation, *N*-glucuronidation, *S*-glucuronidation, and *C*-glucuronidation. *O*-glucuronidation occurs at several functional groups including alcohols (e.g., hexobarbital), phenols (e.g., estrone), carboxylic acids (e.g., α -ethylhexanoic acid; α -aminobenzoic acid), α,β -unsaturated ketones (e.g., progesterone) and hydroxylamines (e.g., *N*-acetyl, *N*-phenyl-hydroxylamine). *N*-glucuronidation occurs on functional groups containing nitrogen such as carbamates (e.g., meprobamate), arylamines (e.g., 2-naphthylamine), aliphatic tertiary amines (e.g., tripropylamine), and sulfonamides (e.g., sulfadimethoxine). *S*-glucuronidation occurs with compounds containing free sulfur group such as aryl thiols (e.g., thiophenol), dithiocarbamic acid (e.g., *N,N*-diethyldithiocarbamic acid) and finally *C*-glucuronidation which occurs very rarely and requires 1,3-dicarbonyl functional groups (e.g., phenylbutazone).

The glucuronidation reaction is catalyzed by glucuronosyl transferase isoforms and require UDP-glucuronic acid as a cofactor. Similar to cytochrome P450 isoforms, multiple glucuronosyltransferases are present with overlapping and distinct substrate specificities. Table 7 presents the known glucuronosyltransferases that catalyze glucuronidation reactions in rats and humans and their tissue distribution.

The disposition profile of glucuronide conjugates in vivo depends on molecular weight. In rats, compounds having molecular weight <250 appear to be predominantly excreted by kidney, whereas compounds having molecular weight >350 appear to be predominantly excreted via bile. Compounds having molecular weights between 250 and 350 appear to be excreted by either pathway (86). Excretion pathways are described later in this chapter.

5.2.1.1. Biological activity and toxicity of glucuronides: Glucuronidation is considered to be a detoxication pathway where lipophilic drugs are converted hydrophilic conjugates and are excreted via bile or urine. However, sometimes, glucuronides are also bioactive either contribute to the pharmacological activity or toxicity. For example, morphine-6-*O*-glucuronide appears to be more potent than morphine itself in its biological activity. Interestingly, considerable species differences exist in morphine glucuronidation. Rats and mice produce inactive morphine-3-glucuronide (M3G),

Table 7 UDP-Glucuronosyl Transferase Isoforms: Species Comparison (Rat Vs. Human)

UDPGT isoforms	Rat		Human	
	Substrate (rat)	Tissue (rat)	Substrate (human)	Tissue (human)
UGT1A1	Bilirubin, estradiol, all-trans-retinoic acid, morphine	Liver	Bilirubin, estradiol, all-trans-retinoic acid, morphine	Liver, bile duct, stomach, colon
UGT1A2	Bilirubin	Liver	—	—
UGT1A2P (pseudogene)	—	—	—	—
UGT1A3	None known	Liver	Low activity, ketoprofen, ®-ibuprofen, (S)-ibuprofen, fenoprofen, naproxen, ciprofibrate, clofibrate, valproic acid, morphine	Liver, bile duct, stomach, colon, kidney, testes, prostate, small intestine
UGT1A4P (pseudogene)	—	—	—	—
UGT1A4	—	—	—	—
UGT1A5	None known	Liver	(R)-Naproxen, (S)-Naproxen	Liver, kidney, skin, bile duct, colon
UGT1A6	(R)-Naproxen; (S)-naproxen	Liver, kidney, duodenum, ovary, testes, epididymis, spleen, lung	(R)-Naproxen, (S)-Naproxen	Liver, kidney, skin, bile duct, colon
UGT1A7	None known	Liver, duodenum, ovary, kidney, testes, spleen, lung	None known	Stomach, esophagus

UGT1A8	None known	—	Androgens; morphine, ciprofibrate, clofibrate, valproate, furosemide, diflunisal, 17-EE, and all- trans-retinoic acid	Esophagus, colon, jejunum, ileum
UGT1A9P	—	—	—	—
UGT1A9	—	—	Fenoprofen, furosemide, ibuprofen, ketoprofen, monoethylhexylthalate, naproxen, retinoic acid, mefenamic acid, 4-catechol estrogens, estradiol, estrol, 2-catechol estrogens	Liver, kidney, esophagus, colon, stomach, small intestine, testes, ovary, mammary gland, prostate, skin, skeletal muscle
UGT1A10	—	—	—	Bile duct, stomach, esophagus, colon, small intestine, jejunum, ileum
UGT1A11P (pseudogene)	—	—	—	—
UGT1A12P (pseudogene)	—	—	—	—
UGT2B1	NSAIDS, valproic acid, clofibrate, ciprofibrate, morphine, estradiol	Liver (low in kidney, lung, intestine, testes)	—	—
UGT2B2	—	Liver	—	—
UGT2B3	—	Liver (low in kidney, lung, intestine, testes)	—	—

(Continued)

Table 7 UDP-Glucuronosyl Transferase Isoforms: Species Comparison (Rat Vs. Human) (*Continued*)

UDPGT isoforms	Rat		Human	
	Substrate (rat)	Tissue (rat)	Substrate (human)	Tissue (human)
UGT2B4	—	—	—	Liver
UGT2B6	—	Liver	—	—
UGT2B7	—	—	NSAIDS, clofibrilic acid, valproic acid, estriol, morphine, estradiol, all-trans-retinoic acid	Liver, kidney, esophagus, small intestine, brain
UGT2B8	—	Liver	—	—
UGT2B10	—	—	—	Esophagus, liver
UGT2B11	—	—	—	Liver
UGT2B12	—	—	—	Liver, kidney, testes
UGT2B15	—	—	Dihydrotestosterone	Liver, testes, prostate, esophagus
UGT2B17	—	—	C19 steroids (androsterone, dihydrotestosterone, testosterone)	Liver, kidney, uterus, placenta, mammary gland, adrenal gland, skin, testes, prostate

whereas humans produce 10–20% of morphine to the morphine-6-glucuronide (M6G). It is also interesting that there are species differences in morphine glucuronidation in vitro. Rat liver microsomes or recombinant UGT2B1 and UGT1A1 produce the inactive M3G, whereas human liver microsomes or recombinant UGT2B7 produces both M3G and M6G. Human brain microsomes also contain UGT activity and express UGT2B7 suggesting that M6G formation from morphine can also occur in human brain.

Codeine, structurally related opioid to morphine, also appears to produce a glucuronide that is active. In addition, steroids (e.g., testosterone, dihydrotestosterone, estradiol, 17 α -ethinylestradiol) containing D-ring glucuronides seem to be cholestatic, whereas the corresponding A-ring glucuronides have the opposite effect (increased bile flow).

Ethinylestradiol, buprenorphine, and lorazepam have been shown to cause jaundice due to their ability to inhibit bilirubin glucuronidation. The glucuronidation pathway of these compounds as well as bilirubin appears to be primarily catalyzed by UGT1A1. Inhibition of bilirubin clearance via glucuronidation by these compounds appears to be responsible for the observed jaundice. Therefore, in toxicology studies, if bilirubin levels in plasma are increased, it should be established whether that drug in question is a substrate and/or inhibitor of UGT1A1.

Reactive acyl glucuronides have received considerable attention over the past decade due to their involvement in toxic adverse reactions. Acylglucuronides of several drugs (e.g., zomepirac, diclofenac, gemfibrozil) are very reactive and form covalent adducts with proteins that appear to be responsible for the toxicities associated with these compounds. Zomepirac was withdrawn from the market due to high incidence of anaphylaxis following drug administration to humans, which has been related to the covalent binding of its acyl-glucuronide (87). Gemfibrozil acylglucuronide can react with DNA, suggesting that this glucuronide is also genotoxic (88). In addition to acyl glucuronides, *N*-*O*-glucuronides of hydroxamic acids such as *N*-hydroxy-2-acetylaminofluorine have also been shown to react with cellular nucleophiles (proteins and DNA), presumably through an arylnitrenium ion (89,90). These reactions have been suggested to play a role in the carcinogenesis of these hydroxamic acids.

Overall, glucuronidation reactions cannot be simply ignored as detoxification pathways and should be regarded as potential mechanisms of bioactivation for biological activity as well as toxicity of drugs. A careful assessment of these reactions should be made in early drug discovery and drug evaluation.

5.2.2. Acetylation

Apart from glucuronidation, glutathione conjugation and acetylation also received some attention due to their toxicological relevance. Acetylation is a detoxification pathway for compounds including aromatic amines. However,

N-acetyl transferases (e.g., NAT1, NAT2) that catalyze this pathway are polymorphic in nature (91). NAT2 is particularly important due to complete deficiency in enzyme activity in a large segment of the population including 50% Caucasians, 10% Asians, and 90% North Africans. Medications metabolized by acetylation include dapsone, procainamide, isoniazid, sulfamethazine, and hydralazine. Of these, procainamide, isoniazid, and hydralazine administration to humans produce systemic lupus erythematosus (SLE; an idiosyncratic immune-disease), due to metabolic activation to reactive intermediates by myeloperoxidase or cytochrome P450 enzymes. It appears that the susceptibility to SLE is dependent on the acetylator phenotype of human subjects, as slow acetylators rapidly develop the disease than faster acetylators (92).

It should also be noted that this NAT-catalyzed reaction generates more lipophilic metabolites (compared to parent drug). This is in contrast to the general role of metabolism in drug clearance (to increase hydrophilicity). For example, procainamide has a narrow therapeutic range and an active acetylated metabolite *N*-acetylprocainamide (NAPA) has a longer $t_{1/2}$ than procainamide. Therefore, both parent and metabolite concentrations of procainamide must be monitored clinically (93).

5.2.3. Glutathione Conjugation and Oxidation

This pathway is an important detoxification system in the body, responsible for conjugating highly reactive substances (epoxides, quinones, products of phase 1). Glutathione (GSH, a tripeptide γ -glutamyl-cysteinyl-glycine) in cells is in millimolar concentrations and therefore can afford protection by scavenging the reactive electrophiles via conjugation (94). If a drug is given at very high doses (e.g., acetaminophen) and all the intracellular GSH is depleted, these reactive electrophilic intermediates covalently bind to cellular macromolecules causing toxicity, death, and genotoxicity. Conjugation of reactive electrophiles with GSH generally occurs on free sulfhydryl group present on cysteine moiety. GSH conjugates are generally further processed to a *N*-acetyl-l-cysteine conjugate (mercapturic acid) which is excreted in the urine. Therefore, *N*-acetylcysteine, which also contains a free sulfhydryl group, is administered as an antidote in cases of acetaminophen overdose. This provides the cell with a replacement conjugating agent (to glutathione), to prevent liver toxicity that results from NAPQI, a reactive metabolite of acetaminophen (generated through CYP2E1). Glutathione conjugation of reactive electrophilic intermediates may or may not require glutathione transferases. For example, quinones rapidly react with glutathione directly, whereas epoxides require a transferase to mediate these reactions. Glutathione transferases are also polymorphic enzymes and the biological significance in drug metabolism has not been explored, although they have been shown to play an important role in environmental toxicology.

Although GSH-conjugation is generally involved in detoxification, it can also produce highly reactive intermediates that covalently interact with macromolecules. Examples include dihaloalkanes, isocyanates, isothiocyanates, and aldehydes (94).

In addition to conjugation of electrophilic intermediates, glutathione can also participate in scavenging and detoxifying free radical metabolites of drugs (95,96). However, in the process, oxygen free radicals may be generated, and thus this reaction may be viewed as a toxic reaction rather than detoxification reaction. Biological monitoring for oxidized glutathione (GSSG; dimer of glutathione) may indicate the extent of free radical reactions. However, these free radical reactions of GSH are complicated, as GSSG can rapidly undergo reduction to GSH by glutathione reductase in the presence of NADPH as the reducing cofactor. Therefore, biological (in vivo) monitoring of free radical reactions of GSH may not be as reliable as GSH-conjugating reactions with electrophiles (these can be excreted as mercapturates in the urine). It is important to note that drugs that undergo biotransformation to free radicals are reduced back to the parent molecule (97,98). Therefore, free radical metabolites of drugs are not easily detectable in vivo.

5.3. Role of Transporters in Drug Metabolism: Synergistic Transport-Metabolism Interactions

It is interesting that CYP3A4, the enzyme that metabolizes more than 50% of the drugs, is also present in the GI tract. The intestinal CYP3A4 accounts for more than 70% of total CYP content in the intestine (99). Recent studies from several laboratories indicate that synergistic transport-metabolism interactions of drugs in the GI tract present interesting aspects of complexity of drug absorption process (99,100). Many Pgp substrates are also CYP3A4 substrates, and therefore drug absorption in the GI tract is dependent on their relative affinities to these two proteins as well as their relative levels of expression. It has been suggested that Pgp in the intestinal epithelial cells can extrude the drug back into intestinal lumen but a portion of the drug is reabsorbed. Several iterations of this process can expose the drug to CYP3A4 also in the gut and results in extensive presystemic gut wall metabolism (100). The best case example of this phenomenon is shown with indinavir, a substrate of both Pgp and CYP3A4 (101). Pretreatment of rats with dexamethasone results in a 2.5-fold increase in both Pgp and CYP3A4 levels, but the first pass metabolism of indinavir is increased sixfold (102). These authors suggest that the 2.5-fold increase in CYP3A1/2 levels alone cannot explain the increased metabolism because the parallel increase in Pgp expression should also increase the extrusion of the drug back into lumen. Therefore, these authors concluded that Pgp-mediated drug efflux

and influx actually increases the residence time of the drug exposure for CYP3A1/2-dependent metabolism in the gut.

Based on the elimination (metabolism and transport) characteristics of drugs, Benet (102a) very recently suggested modifications for the widely accepted BCS classification (based on permeability and solubility). He argues that the modified version of such a classification system may be useful in predicting overall drug disposition, including routes of drug elimination and the effects of efflux and absorptive transporters on oral drug absorption; when transporter-enzyme interplay will yield clinically significant effects (e.g., low bioavailability and drug-drug interactions); the direction, mechanism, and importance of food effects; and transporter effects on post-absorption systemic drug concentrations following oral and intravenous dosing. The importance of this novel concept and its acceptance by regulatory agencies, will certainly be a choice of debate by many scientists in the pharmaceutical industry and academia.

5.4. Active Metabolites

The issue of active metabolites has attracted interest from the pharmaceutical industry due to the complicated role drug metabolism plays in drug toxicity. Recently, a PhRMA committee published a manuscript, which is famously known as the MIST document, that addresses the issue of drug metabolites in safety evaluation and the use of metabolite toxicokinetics in safety assessment (103).

According to MIST, three major points should be considered with regard to metabolites before one incorporates them into a toxicokinetic evaluation: (I) major vs. minor metabolites; (II) pharmacologically active vs. inactive; and (III) metabolites that may have a known toxicity or contain a structural alert for toxicity. The major metabolite is defined as "one that accounts for 25% or more of the exposure to circulating drug-related material" which should be considered for quantification in preclinical toxicology and clinical studies if a need arises. However, this definition was challenged by FDA arguing that it could be misinterpreted as meaning that any metabolite less than 25% of parent drug could be of lesser concern, which may not always be the case (104). For example, reactive metabolites that damage cellular macromolecules are formed in very small quantities but are very important toxicologically. MIST also suggested that if a unique metabolite is formed in humans which is not seen in animal species, the safety evaluation of such metabolite should be handled on a case-by-case basis regardless whether it is the major metabolite or not. Nevertheless, if a metabolite is more active than the parent drug, the quantitation of the metabolite should be considered. This document addressed the use of an *in vitro* species comparison of drug metabolism including humans during

early drug development to enable selection of the appropriate animal model (i.e., similar to humans) for conducting toxicology studies.

5.5. Physiologic Factors Affecting Drug Metabolism

The clearance of an eliminating organ is defined as the volume of perfusing medium that is effectively cleared of drug by that organ per unit time (105). Several models of elimination have appeared in the literature to explain and quantitatively predict the influence of several physiologic factors on drug clearance. These include the venous equilibration model, the sinusoidal perfusion model, and the dispersion model (106). Currently, the most widely accepted is the venous equilibration model. The venous equilibration model or “well-stirred” model (107) assumes the eliminating organ is a single well-stirred compartment through which the concentration of unbound drug in the exiting blood is in equilibrium with the unbound drug within the organ.

The venous equilibration model was developed to quantify changes in hepatic clearance and metabolism when perfusion flow, protein binding, and intrinsic clearance are altered (105,108). Intrinsic clearance is defined as the ability of the liver to clear drug in the absence of restrictions due to blood flow or protein binding. In other words, intrinsic clearance reflects the maximum metabolic or transport capability of the clearing organ.

The extraction ratio is thus defined as the ratio of total drug clearance from an organ to the blood flow supplying that organ (107). Extraction ratio, a measurement of the efficiency of an organ in removing drug, is the fraction of drug removed with each pass of blood through that organ. The hepatic extraction ratio can range from 0 to 1. Accordingly, the maximum value of clearance is hepatic blood flow.

The extraction ratio (E) of a drug can be classified as *restrictive* or *non-restrictive*. This classification is based upon the dependence of drug clearance on protein binding in the circulation. Generally, the clearance of a high extraction compound ($E > 0.7$) is nonrestrictive; that is, the eliminating organ is capable of extracting the entire amount of drug presented to it regardless of the degree of protein binding. In these cases, the clearance approaches a maximum value, the blood flow to the organ. Hence, the elimination of a high extraction compound is sometimes referred to as *perfusion rate-limited*. Conversely, the opposite is observed for a compound with a low extraction ratio. The ability of the eliminating organ to remove drug is limited by the dissociation of drug from blood proteins. Such compounds are referred to as restrictively cleared and elimination is dependent upon the free fraction of the drug in the blood (and the intrinsic clearance).

5.5.1. Intrinsic Clearance

As stated above, intrinsic clearance reflects the true metabolizing capability of the liver. Compounds with high intrinsic clearances fall into the high

extraction ratio classification. When dosed orally, the compounds undergo significant first-pass metabolism, resulting in poor bioavailability. Moreover, significant drug interactions result from induction and inhibition of liver enzymes, since changes in enzyme expression and or activity affect intrinsic organ clearance.

Prediction of intrinsic clearance for a drug, prior to testing in man, is useful in selecting agents with the least significant pharmacokinetic liabilities for development. Two methods have been widely used to calculate Cl_{INT} from in vitro data: the kinetic analysis method (109) and the half-life method (110). The former method involves the determination of enzyme kinetic parameters, and the latter involves the measurement of the first-order rate constant for consumption of substrate at a low concentration. These methods are summarized below.

5.5.1.1. Kinetic analysis method: Intrinsic clearance (Cl_{INT}) is based on a classical biochemical kinetic process and can be calculated from the rate of metabolism (dX/dt) and drug concentration (C):

$$Cl_{INT} = \frac{dX/dt}{C} \quad \text{or} \quad \frac{dX}{dt} = Cl_{INT} \times C \quad (1)$$

When the rate of metabolism is expressed in terms of Michaelis–Menten kinetics:

$$\frac{dX}{dt} = \frac{V_{\max} \times C}{K_m + C} \quad (2)$$

where $V_{\max}$ is the maximum rate of metabolism and K_m is the Michaelis–Menten constant (concentration where rate = $\frac{1}{2} V_{\max}$) for the drug–enzyme interaction. Under linear (i.e., first order) conditions of metabolism, when drug concentrations are less than 10% of K_m , Eq. (2) can be written as

$$\frac{dX}{dt} = \frac{V_{\max}}{K_m} \times C = Cl_{INT} \times C \quad (3)$$

Therefore, intrinsic clearance can be estimated from the Michaelis–Menten parameters in vitro.

5.5.1.2. $t_{1/2}$ method: The $t_{1/2}$ values following in vitro incubations of drug and microsomes can be calculated from the slope of a plot of log percentage drug remaining vs. incubation time (slope = $-k = 0.693/t_{1/2}$). The $t_{1/2}$ value can be converted to Cl_{INT} by the following equation:

$$Cl_{INT} = \frac{0.693}{t_{1/2}} \times \frac{ml_{\text{incubation}}}{mg_{\text{microsomes}}} \quad (4)$$

5.5.2. In Vitro-In Vivo Correlations of Cl_{INT} Determinations

Several review articles have been published assessing the value of in vitro clearance data in predicting the in vivo clearance (111,112). A general strategy is to first conduct in vitro incubations and determine the kinetic parameters (V_{max} , K_m) and calculate the in vitro Cl_{INT} for the formation of each metabolite. In the second step, appropriate scaling factors are used to convert in vitro units ($\mu\text{g}/\text{min}/\text{mg}$ protein or number of cells) to in vivo units ($\mu\text{L}/\text{min}/\text{SBW}$, where SBW is standard body weight). In the third step, different models are used to predict hepatic Cl from the observed Cl_{INT} . These three steps may be sufficient to predict the in vivo clearance of drug. However, in some cases, additional modeling may be required if the drug is cleared by nonhepatic routes or by extra-hepatic metabolism.

Several in vitro hepatic models including liver microsomes, hepatocytes, and liver slices have been used in predicting the in vivo clearance of drugs. Studies from several laboratories indicated that hepatocytes are a superior model compared to liver microsomes or liver slices in predicting the in vivo clearance (5,109–113). Liver microsomes are also good predictors, but several scaling factors need to be included such as protein binding, end product inhibition, etc. However, the scaling factors may not be consistently the same for every drug, and modifications may be required case by case. Atypical (non-Michaelis–Menten) kinetics by CYP enzymes can present a problem in accurate predictions (109). In addition, if a drug is primarily metabolized by phase 2 glucuronidation pathways, then microsomes represent a poor model unless fortified by appropriate cofactors to produce glucuronide metabolites. In this regard, hepatocytes may represent a better tool because they produce both phase 1 and phase 2 metabolites and do not require supplementation of appropriate cofactors. However, due to limited supply of human hepatocytes, microsomes are still preferred model for carrying out in vitro metabolism studies. Nevertheless, recent advances in cryo-preserved technology seem promising for expanding the availability of human hepatocytes for in vitro studies in the future. Liver slices are a poor predictive model due to the diffusion problems and unequal distribution of the drug into the slices. Haenen et al. (114) suggested that liver slices may be useful tool for compounds with low extraction efficiency.

Recently, Clarke and Jeffrey (115) retrospectively evaluated 1163 compounds from 48 chemistry programs to determine the in vitro–in vivo correlation of clearance predictions using in vitro rat liver microsomal data and in vivo rat data. Their data show that ~64% compounds were correctly predicted. Roughly 24% of compounds were metabolically stable in vitro but had clearance values exceeding 50% liver blood flow in vivo in the rat. This suggests noncytochromeP450 mediated pathways may metabolize these compounds. The remaining 12% compounds had in vivo clearance values less 50% liver blood flow, yet were metabolically unstable in vitro. It appears

that these compounds have high protein binding, short half-lives and inhibitory properties against the major cytochrome P450 isoforms. These results suggest that *in vitro* methods can be applied to predict *in vivo* metabolic clearances but should be carefully evaluated for false positives or false negatives (particularly the latter). Surprisingly, in this study, human liver microsomes compared favorably with rat liver microsomes for predicting *in vivo* clearance in rat, although one would expect species differences in *in vitro* metabolic clearance.

5.6. Metabolic Drug Interactions

A major concern in drug development involves assessment of the potential for drug interactions involving a new compound. This includes drug–drug, drug–food, and drug–disease interactions. From the perspective of drug development, it is important to identify potential drug–drug interactions for a new chemical entity early on (preclinical screening) before clinical studies are conducted.

In general, many reported drug interactions are not of clinical significance (116). Factors to consider when evaluating the likelihood of a clinically important drug interaction include the therapeutic index of the affected drug, the likelihood for coadministration of the drug and interacting agent in patients, and the effect of the interaction on the clearance of the drug (and therefore plasma levels). While drug interactions have been identified for numerous ADME processes, interactions that result in changes in drug clearance are the most important.

Metabolic drug interactions are of two general categories: enzyme inhibition and enzyme induction. Each of these is described below.

5.6.1. Enzyme Inhibition

Enzyme inhibition is a fairly rapid process; that is, drug metabolism is affected quickly upon systemic exposure to an interacting drug. Metabolic inhibition can lead to drug toxicity due to elevated plasma levels secondary to reduced clearance. There are several mechanisms of enzyme inhibition: competitive inhibition, noncompetitive inhibition, and irreversible inhibition. Detailed information regarding mechanisms of enzyme inhibition (and methods to study enzyme inhibition) can be found in the literature (117,118).

With regard to CYP, the list of inactivators for CYP isozymes is fairly well established. Examples of CYP inhibition for therapeutic use include CYP19, an enzyme responsible for estrogen production (119). A possible therapeutic target of estrogen-dependent tumors is irreversible inactivation of this enzyme. Also, the mechanism of action of ketoconazole is inhibition of CYP51, an enzyme involved in lanosterol 14-demethylation and the pathogenesis of fungal infections) (119). For cancer prevention, inhibition

of CYP1A1 may be important since the induction of this system may be a risk factor for carcinogenesis (119).

CYP3A4 is the major form of P450 expressed in normal adult human liver and accounts for ~30–50% of the total P450 content in the human liver microsomes and in intestinal gut wall enterocytes, respectively. This enzyme is also the major isoform involved in drug metabolism, accounting for metabolism of more than 50% of the known drugs on the market. Therefore, CYP3A4-related drug interactions are a major concern during drug development. For example, the calcium channel blocker Mibefradil (Posicor®) was withdrawn from the market because of its potential to inhibit CYP3A4, thus resulting in metabolism-based drug interactions (120). Terfenadine (Seldane®), a H₁-receptor antagonist and CYP3A4 substrate, was also withdrawn from market because its metabolism was inhibited by several CYP3A4 inhibitors, resulting in fatal cardiac arrhythmias (116,121).

Predictions of CYP3A4-mediated clinical drug interactions from in vitro systems are still at qualitative level, and are typically used as confirmatory studies to guide clinical drug interaction studies. However, in a few cases, quantitative predictions were achieved. Recently, PhRMA published a manuscript detailing the in vitro experimental conditions that should be used for predicting clinical drug–drug interactions, in an attempt to standardize/harmonize the process across the pharmaceutical industry (117). In particular, experimental results (e.g., K_i values) may be overestimated when high concentrations of microsomal protein are utilized. Under these conditions, the inhibitor may bind nonspecifically to microsomal proteins and thus is not available to inhibit the metabolism of the drug in question. This has been demonstrated for several CYP3A4 inhibitors including ritonavir, ketoconazole, itraconazole, fluvoxamine, and norfluoxetine. For example, inhibition of diazepam (100 μ M) metabolism to temazepam in human liver microsomes was decreased as the protein concentration was raised from 0.65 to 3.0 mg/mL, when fixed concentrations of CYP3A4 inhibitors were used (122).

Overall, successful prediction of clinical drug interactions may be obtained using therapeutically relevant concentrations of the substrate and the inhibitor (117). The use of very high concentrations of drug or inhibitor may produce drug interaction in vitro, which is not observed in vivo.

5.6.2. Enzyme Induction

Induction is an increase in enzyme activity associated with an increased intracellular enzyme concentration. The effect is generally dose dependent and the duration of exposure to inducing agent can vary from 2 days up to 2 weeks (123). Also, the inducing effect takes time to dissipate once the inducing agent is removed. Enzyme inducers are not only medications but can also be obtained through diet (e.g., alcohol) and the environment (e.g., smoking). The result of induction is an increased metabolism and therefore increased clearance. It is important to note that induction is only

one factor that contributes to intersubject variability in metabolism. One report suggests that intersubject CYP3A4 capability varies 15–100-fold (124).

Many CYP isozymes (e.g., CYP1A1/1A2, CYP2B6, CYP2E1, CYP2C9, CYP2C19, CYP3A4) are inducible and as such enzyme induction may contribute to changes in drug clearance and drug toxicity. Interestingly, CYP2D6 is not an inducible enzyme.

CYP enzyme induction may occur by several mechanisms including increased gene transcription, mRNA, and/or protein stabilization (125). CYP1A inducers act by binding to a cytosolic AhR (aryl hydrocarbon receptor). This receptor drug complex then undergoes heterodimerization with Ah nuclear translocator (Arnt) protein in the nucleus. This Arnt–AhR–Drug complex binds to the enhancer region in xenobiotic responsive element (XRE) and acts as a transcription factor thus inducing the transcription of CYP1A gene. Similarly CYP3A inducers act by binding to a nuclear PXR (Pregnane X Receptor), which forms a heterodimer with RXR (retinoic acid receptor). This PXR/RXR complex is activated by CYP3A inducers and bind to the promoter region of CYP3A gene resulted in increased transcription of CYP3A gene. CYP enzyme induction in some cases may not necessarily be mediated by enhanced gene transcription. For example, CYP2E1 enzyme induction occurs by mRNA or protein stabilization as it occurs during alcohol consumption or during starvation, respectively.

Whether or not induction (or inhibition) is important clinically depends on a number of factors (discussed previously). Since many medications are metabolism via several pathways that are under control of different enzymes, inhibition of one pathway will result in compensation by other pathways. On the other hand, induction of a potentially toxic pathway is problematic as seen with the effect of alcohol on acetaminophen toxicity (126).

CYP1A2 induction by omeprazole (a proton pump inhibitor) has been associated with severe side effects such as complicated vision disturbances (127,128). Similarly, CYP3A4 induction by troglitazone (an insulin sensitizer) has been associated with hepatic dysfunction and hepatic failure in humans (129,130). Induction of CYP3A4 may also result in drug interactions resulting in loss of efficacy another drug by inducing drug. For example, rifampin, an antituberculosis drug is a potent inducer of CYP3A4 in humans and is known to decrease the bioavailability and efficacy of several drugs (reviewed in Ref. 123). These include analgesics, antidiabetics, antiepileptics, psychotropics, antimicrobials, antifungals, cardiovascular, anticoagulants, hormones, and immunosuppressants. For example, rifampicin coadministration with HIV-protease inhibitors (nelfinavir, indinavir, saquinavir) is contraindicated. Rifampin also decreases the efficacy of oral contraceptives and can result in acute transplant rejection in patients treated with immunosuppressive drugs (e.g., cyclosporin). Similarly, several other CYP3A4 inducing antiepileptic agents increase the CYP3A4 metabolism of oral contraceptives (estrogens and corticosteroids) and decrease their

contraceptive potency (131). Therefore, determining the induction potential of the candidate drug early in drug development is crucial for predicting the drug-related toxicities and drug interactions.

Until recently, *in vivo/ex vivo* animal induction models were used to determine the CYP induction potential of drugs. However, due to species differences in induction profiles, the relevance of the induction data obtained in animals to humans has been questioned. For example, dexamethosone is a potent inducer of CYP3A1 in rat hepatocytes, while rifampin is not (132). However, rifampin is a potent inducer of CYP3A4 in human hepatocytes and CYP3A6 in rabbit hepatocytes. These effects of dexamethasone and rifampin are also reproduced in rats and humans *in vivo*. Therefore, human hepatocytes appear to be a better model to determine the CYP enzyme induction potential in humans.

Ex vivo induction in animal models is important for careful planning and designing long-term regulatory-driven toxicology studies. If there is a rapid auto-induction of metabolism in animal models, then determining the safety margins for the parent compound can be complicated. In this case, higher doses of drug should be administered in preclinical situations to compensate for the lost drug due to metabolism. However, this can also result in exacerbated toxic effects, particularly if the metabolite is the toxic species. For example, CYP2E1 induction by ethanol can increase acetaminophen hepatotoxicity due to increased formation of reactive and toxic quinoneimine metabolite.

In some cases, carcinogenic responses noted in animals due to enzyme induction are not relevant to humans. Two widely cited examples are induction of CYP4A and UDPGT enzymes. Induction of CYP4A enzymes in rodents is involved in peroxisomal proliferation and, subsequently, hepatocarcinogenesis (133). However, primates and humans appear to be either nonresponsive or very poor at inducing CYP4A and peroxisomal proliferation. Thus, a rodent hepatocarcinogen with associated peroxisomal proliferation may not result in similar effects in humans.

Similarly, chemical-mediated thyroid tumor formation in rodents has been related to the induction of UDP-glucuronosyl transferases which glucuronidate endogenous thyroxine and triiodothyronine (134,135). In rat liver UGT1A1, 1A6 and UGTB2 are responsible for glucuronidation of thyroxine and triiodothyronine, respectively (136,137). In humans, UGT1A1 and 1A9 have been shown to glucuronidate thyroxine and triiodothyronine. A rapid and excessive glucuronidation of thyroxine in the liver, followed by extensive biliary excretion of these glucuronides, results in reduced circulating levels of thyroid hormones. Consequently, prolonged elevation of serum thyroid stimulating hormone can occur, promoting thyroid tumor formation. Although chemically mediated increased thyroid hormone metabolism and reduction of circulating thyroid hormone levels were also detected in humans, they appear to be less susceptible for thyroid

tumor development from thyroid hormone perturbations. The underlying mechanisms for these different sensitivities between rats and humans are not yet clear, although human UGT1A1 and 1A9 are inducible (137).

5.6.3. Identification of CYP Enzymes Involved in Drug Metabolism

In addition to enzyme inhibition and induction studies, successful prediction of metabolic drug interactions from *in vitro* data also requires identification of the human CYP isozymes responsible for the metabolism of the test drug, particularly when other compounds can inhibit the metabolism of the test drug (117). However, if a drug is metabolized by more than one CYP isoform, then the metabolism of the test drug may be compensated even if one CYP isoform is inhibited. Therefore, the relative contribution (by determination of kinetic parameters) of the metabolic pathways by different isozymes should be determined. There are three main approaches utilized in CYP isozyme identification in human liver microsomes (117,138,139): (I) metabolism using cDNA expressed enzymes; (II) effect of specific chemical inhibitors or antibodies of CYP isoforms, on the metabolism of the test drug; (III) correlation analysis with specific probe substrate metabolism using a panel of human liver microsomes pooled from 10 to 15 subjects.

6. MECHANISMS OF SMALL MOLECULE EXCRETION

6.1. Renal Excretion

The kidney is the primary organ responsible for the excretion of medications and their biotransformation products from the body. Detailed reviews of renal drug excretion mechanisms are available in the literature (140,141). The major processes involved in the renal elimination of drugs are glomerular filtration, active tubular secretion, intrarenal metabolism, and passive reabsorption. The combined effect of the first two processes is the extraction of drug from the blood into the urine. The last process, reabsorption, involves the movement of drug back into the blood from the primitive urine. Thus, the renal excretion rate of a compound is the net result of these individual mechanisms.

6.1.1. Glomerular Filtration

Urine formation begins with glomerular filtration. The glomerular filtrate normally contains no cells, is essentially protein-free, and contains most inorganic ions and low-molecular weight organic solutes (glucose and amino acids, for example) in virtually the same concentrations as in the plasma. The quantity of drug that is filtered by the kidney parallels the concentration of unbound drug in the plasma. Overall, the rate of filtration is the product of unbound plasma concentration and glomerular filtration rate (GFR) (142).

6.1.2. Tubular Secretion

Once the plasma is filtered and ultrafiltrate enters the nephron, several forces operate to alter the concentrations of assorted substances in that fluid and, ultimately, the mass of each that is excreted. Although most compounds that are renally eliminated undergo glomerular filtration, the extraction of compound via this mechanism is relatively low, particularly if the compound is highly protein bound. A second mechanism by which drug is extracted from the blood into the urine is tubular secretion; that is, the compound is transported from the blood across the kidney tubule cell into the urine.

Tubular secretion is an active, carrier-mediated process that occurs in the proximal tubule. A number of membrane transporters have been identified in the kidney. Over the past decade, significant progress has been made in the cloning, functional expression and initial characterization of these transporters. To date, five organic cation transporters and nine organic anion transporters have been cloned (143). These transport systems are detailed in [Chapter 8](#).

Membrane-bound transporters are responsible for the translocation of xenobiotics across the basolateral and luminal membranes of the kidney cell (143,144). Consequently, active tubular transport contributes to the cellular accumulation and urinary excretion of medications. Additionally, these transporters are potential sites for significant drug–drug interactions *in vivo*.

Analogous to metabolic clearance, the venous equilibration model is also applied to renal drug excretion. Intrinsic renal clearance (Cl_{INT}) is defined as the clearance of a drug in the absence of flow and binding restrictions (142). For compounds of low renal extraction (renal clearance is small relative to kidney plasma flow), clearance is considered restrictive (protein binding dependent). Conversely, the renal clearance of high extraction compounds is nonrestrictive (flow dependent). Such compounds (e.g., para-aminohippuric acid) are excellent substrates for the secretory transport system and are capable of being almost completely extracted from the blood regardless of the degree of protein binding. For this reason, the renal clearance of para-aminohippuric is used as a marker for renal plasma flow.

Proximal tubular secretion is inferred when the rate of excretion of a particular compound exceeds the rate of filtration (142). Since it is carrier-mediated, proximal tubular secretion is a saturable process and accordingly, the kinetics of the secretion can be described by Michaelis–Menten (140).

6.1.3. Tubular Reabsorption

While filtration and secretion systems in the kidney serve to eliminate drug from the blood into the urine, tubular reabsorption serves to counteract excretion from the blood. Active reabsorption occurs for many endogenous compounds (i.e., glucose, electrolytes). The identification of transport systems in the luminal membrane of the kidney cell (e.g., peptide transporters,

nucleoside transporters) suggests a role of active transport in drug reabsorption by the kidney. However, the predominant mechanism of drugs reabsorption is passive transport.

The primary driving force for passive reabsorption is the tubular reabsorption of water, which serves to concentrate the drug in urine with respect to plasma (142). It is the establishment of this electrochemical gradient that allows for back diffusion of drug molecules from primitive urine to blood. The degree of reabsorption is dependent upon physicochemical properties of the drug and physiologic variables. The physicochemical properties include polarity, state of ionization, and molecular weight. Small, nonionized, lipophilic molecules tend to be extensively reabsorbed (145). Physiologic variables that affect reabsorption include urine pH and urine flow rate. Generally, increasing the urine flow rate tends to decrease the concentration gradient, contact time, and, subsequently, the extent of reabsorption. Additionally, if the drug is weakly acidic or basic, perturbations in urine pH will influence reabsorption.

When total renal clearance is less than the clearance due to filtration (reabsorption must be occurring). In general, however, it is difficult to accurately quantify the reabsorption of a compound. Equations have been proposed to estimate reabsorptive clearance (146–148), but are rarely accurate in predicting observed clearances (149). A particularly useful renal clearance parameter is excretion ratio. Excretion ratio (*XR*) is simply the renal clearance corrected for filtration clearance:

$$XR = \frac{Cl_{\text{renal}}}{f_u \times GFR} \quad (5)$$

where Cl_{renal} is the overall renal clearance of the medication and f_u represents the fraction of drug unbound in the plasma (determined experimentally). An excretion ratio value greater than one is indicative of a net secretory process. Conversely, an excretion ratio less than one is reflective of a net reabsorptive process. Therefore, an estimation of excretion ratio provides a general indication of the mechanism of elimination for the compound of interest.

6.1.4. Renal Metabolism

The liver accounts for the majority of drug metabolism, although for some drugs the kidney accounts for 15–40% of total metabolism (150). Because most studies focus on the role of the liver in drug metabolism, there is relatively sparse literature on renal drug metabolism. Generally, identification of a highly concentrated urinary metabolite not present in plasma is suggestive that the formative site is in the kidney. Most often the kidney contributes to the phase 2 metabolism of a drug, although weak immunoreactivity to CYP3A4 and strong immunoreactivity to CYP9 has

been identified in the kidney (151). Major reactions shown to occur in the kidney include *N*-glucuronidation, sulfation, and glycation. The net result of renal drug metabolism is an increase in the polarity of a drug, thereby increasing the probability for excretion into the urine.

6.2. Biliary Excretion and Enterohepatic Recycling

Although widely recognized as the major site of drug biotransformation, one of the main functions of the liver is the formation of bile. Hepatic xenobiotic disposition involves a number of different pathways including uptake into the hepatocyte, intracellular translocation, biotransformation and egress into blood, and/or bile.

Biliary excretion contributes to the disposition of a number of endogenous substances as well as medications. As detailed in [Chapter 8](#), studies with knockout animals have demonstrated that biliary secretion of medications is mediated by at least two transport systems: Pgp and MRP2 (formerly known as cMOAT). Biliary excretion depends on a number of factors including chemical structure and molecular weight. In humans, the molecular weight threshold for biliary excretion is 600 (152). For compounds with molecular weights <600, the primary route of excretion from the body is renal elimination. In rodents, the molecular weight cutoff is 350 for biliary excretion as described earlier.

Enterohepatic recycling involves a sequence of events beginning with drug removal from the circulation by the liver and secretion into bile (in some cases after metabolism). Drug (or metabolite) is transported via the bile into the duodenum, where it is subsequently available for reabsorption back into the circulation. In some cases, reabsorption follows intestinal deconjugation by intestinal bacteria. Accordingly, enterohepatic recycling depends on a number of factors that are associated with each of these processes (absorption, metabolism, excretion). The consequences of enterohepatic recycling include a prolonged residence of drug in the body (i.e., longer $t_{1/2}$), and the possibility of multiple peaks (in the plasma concentration-time profile) following oral administration.

An excellent review of the topic of enterohepatic recycling was recently published (152). Among the medications known to undergo enterohepatic recycling include pain medications (e.g., morphine, NSAIDs), antibiotics, and hormones. Additionally, enterohepatic recycling of a medication is affected by disease, genetics, and coadministration of other compounds.

6.3. Excretion into Breast Milk

Although medication use among lactating women generally poses limited risk to a breast-fed infant, excretion of medication into breast milk has potential clinical and toxicological implications (153). There are a number of experimental methods (both in vitro and in vivo) and models to assess

drug excretion into breast milk, and for determining the potential risk to a nursing infant (154).

Traditionally, drug excretion into breast has been traditionally thought of a passive process, and physicochemical properties of the drug and regional differences in pH (breast milk is more acidic compared to plasma) dictate the concentration of drug in milk. However, recent evidence suggests that membrane transporters in the mammary gland are also involved in drug transfer into breast milk (155). The relative contribution of these carrier-mediated transport systems remains to be elucidated.

7. SMALL MOLECULE ADME: ISSUES FOR DRUG DEVELOPMENT

7.1. Pharmacogenetics

Today, identification of genetic differences in drug metabolism among different ethnic groups plays a pivotal role in clinical studies to understand the variability in pharmacokinetic variability in humans. As described previously in this chapter, the impact of pharmacogenetics on drug metabolism is illustrated by the established polymorphisms of the CYP family and *N*-acetyltransferase. Likewise, polymorphism in membrane transport proteins is expected. While there is limited information on the pharmacogenomics of drug transporters in general, the effect of polymorphism on Pgp expression and function is the subject of considerable interest.

Polymorphisms of Pgp in humans have recently been documented with at least 16 SNPs (single nucleotide polymorphisms) identified to date. It is anticipated that more SNPs will be identified in future. Interestingly, most of these polymorphisms (although resulting in amino acid changes of the protein) are silent. However, variable expression of Pgp mRNA and protein levels in the gut has been demonstrated, with an estimated 8–10-fold variation. Besides genetic factors, the variability of Pgp expression in humans may also depend upon environmental factors such as diet. Clearly, much more needs to be unraveled regarding the structure–function relationships involving Pgp and other transporter families.

7.2. Species Differences in Drug Disposition

7.2.1. Species Differences in Drug Metabolism

Species variation in drug metabolism is well established and has led to the identification and characterization of the enzymes involved and their differences in catalytic activities across several animal species (and strains) including man.

Early studies with hexobarbitone (72) in several animal species have shown good pharmacokinetic–pharmacodynamic relationship among $t_{1/2}$,

Table 8 Species Differences in Hexobarbitone Sleeping Time, Half-Life and Metabolism: An Example of Pharmacokinetic–Pharmacodynamic Correlation

Species	Sleeping time (min)	Hexobarbitone half-life (min)	Hexobarbitone metabolism (units)
Mouse	12 ± 8	19 ± 7	16.6
Rat	90 ± 15	140 ± 54	3.7
Dog	315 ± 105	260 ± 20	1
Man		~360	

metabolism, and sleeping time in mouse, rat, and dog (Table 8). $t_{1/2}$ and sleeping time were inversely related to metabolism.

With regard to the drug metabolism, species differences have been shown for CYP activity. For example, metabolite profiles of caffeine are quite different in rat, rabbit, monkey, and human, which perhaps reflect species variations in CYP1A2, the primary isoform responsible for caffeine *N*-demethylation. Coumarin, a CYP2A substrate, is metabolized in rat to 3,4-epoxide which subsequently undergoes ring opening to *o*-phenylhydroxyacetaldehyde as the reactive toxic species. However, in humans, coumarin is primarily metabolized by CYP2A6 to 7-hydroxycoumarin which is considered to be a detoxification pathway (156). In addition, rat CYP2A1 and CYP2A2 catalyze 7 α or 15 α hydroxylation of testosterone, but human CYP2A6 does not catalyze these reactions (157). CYP2B isoforms in rat and dog hydroxylate androgens at 16 α and 16 β positions, whereas guinea pig and monkey CYP2B isoforms catalyze hydroxylation exclusively at 16 β position and the rabbit exclusively at 16 α position (158,159).

The CYP2C subfamily also shows marked variation in regio- and stereoselectivity of steroid metabolism between different species and also exhibits differences between male and female, which can be ascribed to gender-specific isoforms (160). Species differences in stereoselective 4'-hydroxylation of *S*- and *R*-mephenytoin have been demonstrated noted in studies using liver microsomes of mice, rats, dogs, rabbits, monkeys, and humans (161). Humans (CYP2C9) and monkeys preferentially catalyzed *S*-mephenytoin 4'-hydroxylation (160). Conversely, rats (CYP2C11), rabbits, and dogs catalyzed *R*-mephenytoin 4'-hydroxylation at rates 2–6-fold higher rates than *S*-mephenytoin 4'-hydroxylation. However, hydroxylation of both isomers occurred at similar rates in mice. Bogaards et al. (161) concluded that none of the cytochrome P450 activities in the animal species are similar with respect to humans; however, for all the CYP activities but in each species some of the CYP activities can be considered similar to man.

Species differences have also been noted with phase 2 conjugation pathways. For example, phenol is metabolized by conjugation to glucuronide and/or sulfate, and the relative proportion of these two metabolites

depends on the species studied (72). Hepatic metabolism can reduce the bioavailability of drugs even when 100% of the drug is absorbed through GI tract. In such cases, the bioavailability of drugs may be estimated as a total of drug and its metabolites. However, considerable species differences exist in substrate specificities of CYP isoforms. Table 9 shows the differences in catalytic activities of dog and human liver microsomes for specific CYP substrates. As shown in the table, dog liver microsomes catalyzed coumarin 7-hydroxylase and testosterone 6 β -hydroxylase activities at rates several fold higher than to human liver microsomes (162). Therefore, such species differences in drug metabolism complicate the prediction of pharmacokinetics in humans from preclinical animal studies. In addition, hepatic drug metabolism by cytochrome P450 enzymes is very complex as these enzymes exhibit non-Michaelis–Menten kinetic behaviors due to very complicated biochemistry (163). Efforts to predict in vivo hepatic drug clearance and drug–drug interactions from in vitro methods were largely unsuccessful primarily due to these issues.

7.2.2. Species Differences in Membrane Transport

Membrane transporters play a critical role in drug disposition. Mechanisms of absorption, distribution, metabolism, and excretion are mediated by organ uptake of xenobiotics. Humans and other animal rodent membrane transporters are not orthologous; that is, amino acid sequences of transport proteins are different among between species. Species differences in transporter expression and orthology are an important issue for preclinical drug development. Future research will undoubtedly determine differences in transporter activity (and substrates) among species. Extrapolation of whole animal studies to predict clinical outcomes will depend on the extent of overlap in transporter expression and activity between species (164).

7.3. Other Factors Affecting Drug Disposition

7.3.1. Gender

Gender effects on drug disposition are another emerging issue for drug development. The influence of gender on pharmacokinetics and drug activity is well established, and has been the subject of recent reviews (165,166). Differences in pharmacokinetics between males and females are the result of biological differences between genders. These differences include body weight, body composition, and hormonal. Additionally, it appears that sex-related differences in drug metabolism and membrane transporters are an underlying cause of these differences (167).

While gender-specific variations in drug response have been demonstrated for several medications, there has been paucity of research in this area. There is an increasing need for clinical studies that emphasize the role of gender on pharmacokinetics and pharmacodynamics. Although there is

Table 9 Species and Gender Comparison of Cytochrome P450 Catalytic Activities (nmol/min/mg): Human Vs. Beagle Dog Liver Microsomes

Catalytic activity measured	Human male	Human female	Human mean	Dog male	Dog female	Dog mean
Phenacetin <i>O</i> -deethylase (CYP1A)	0.615 ± 0.757	0.273 ± 0.212	0.510 ± 0.641	0.748 ± 0.144	0.727 ± 0.128	0.737 ± 0.122
Coumarin 7-hydroxylase (CYP2A)	0.355 ± 0.400	0.572 ± 0.164	0.422 ± 0.352 ^a	0.047 ± 0.014	0.099 ± 0.041	0.073 ± 0.040
Tolbutamide hydroxylase (CYP2C)	0.04 ± 0.03	0.05 ± 0.03	0.04 ± 0.03 ^a	0.009 ± 0.004	0.005 ± 0.005	0.007 ± 0.005
<i>S</i> -Mephenytoin 4'-hydroxylase (CYP2C)	0.035 ± 0.043	0.033 ± 0.046	0.035 ± 0.042	0.043 ± 0.008	0.027 ± 0.012	0.035 ± 0.013
Dextromethorphan <i>O</i> -Demethylase (CYP2D)	0.120 ± 0.076	0.134 ± 0.029	0.125 ± 0.064	0.056 ± 0.010	0.048 ± 0.016	0.052 ± 0.014
Chlorzoxazone 6-Hydroxylase (CYP2E)	0.202 ± 0.119	0.216 ± 0.100	0.206 ± 0.109	0.229 ± 0.075	0.272 ± 0.048	0.253 ± 0.063
Testosterone 6β-hydroxylase (CYP3A)	1.45 ± 1.67	2.18 ± 0.26	1.69 ± 0.66 ^a	0.325 ± 0.111	0.182 ± 0.038	0.254 ± 0.108

^aStatistically different from human.

an emerging body of literature evaluating gender effects on drug disposition, differences in disposition between males and females are not well defined. Elucidation of sex differences in pharmacokinetics is complicated by underlying patient factors including genetics, age, and disease. Consequently, there is a need for better understanding of the role of gender on the disposition and activity of xenobiotics.

7.3.2. Disease

As noted above, the presence of underlying disease can alter the pharmacokinetic profile of a medication (16–18). Of particular importance are diseases that affect kidney and liver function, the two organs responsible for drug clearance. Additionally, alterations in cardiac output and levels of circulating proteins can also affect drug disposition through alterations in organ blood flow and plasma binding, respectively.

The discovery that several endogenous cytokines such as interferons, interleukins decrease CYP enzyme levels and activities suggested that disease state could have profound influence on drug metabolism (168–170). Indeed, bacterial infections have been shown to result in impaired drug clearance in humans (171). Fourteen subjects with acute pneumonia of diverse etiology all had decreased antipyrine clearance during infection. Human volunteers given bacterial LPS showed reduced clearance of antipyrine, hexobarbital, and theophylline that correlated with the initial peak values of tumor necrosis factor and interleukin-6. The involvement of cytokines in altering drug metabolism was later directly demonstrated in studies where humans were given recombinant human IFN α that reduced the clearance of antipyrine, and erythromycin (172,173). These studies indicated that the disease states that alter the endogenous cytokine levels could alter drug metabolism perhaps having an effect on CYP enzymes and their expression.

In terms of renal excretion, changes in kidney function are often assessed through GFR or its clinical marker, serum creatinine. The Whole Nephron Hypothesis assumes that reductions in renal filtration (reflected in GFR) are accompanied by parallel diminutions in secretory and reabsorptive capacity of the nephron (174). Consequently, for medications eliminated primarily via renal mechanisms, dosage adjustments in patients with renal dysfunction are frequently based on GFR (175).

A recent clinical study demonstrated that, in patients with renal diseases, expression of organic anion membrane transporters (OATs) correlated with reduced drug secretion (176). It is likely that future studies will further define the impact of disease-induced alterations in transporter expression of drug disposition.

7.3.3. Ageing

The biological and physiological consequences of aging can dramatically affect the pharmacokinetic profile of a medication (177). This can have serious implications for pharmacotherapy in the elderly. For example, increased gastric pH, decreased GI motility, and reduced intestinal blood flow can affect the rate and extent of drug absorption following oral administration. The shift in body composition (increased body fat, reduced total body water) with age can affect V_D and $t_{1/2}$ of both lipophilic ($\uparrow$ distribution) and hydrophilic ($\downarrow$ distribution) compounds. Likewise, alterations in cardiac output, organ mass, and organ function collectively reduce the ability of aging individuals to clear medications. The pharmacokinetics of a drug is also subject to changes in pediatric subjects, where drug metabolism enzyme expression is considerably different from adult humans (178,179). In recent years, considerable research is devoted in understanding the pharmacogenomics of drug metabolizing enzymes in children also (180). Several other factors related to absorption, distribution, and excretion could also contribute to pharmacokinetic differences in children (181).

The expression of CYP enzymes changes markedly during development. CYP3A7 is the predominant CYP isoform expressed in fetal liver. CYP3A7 peaks shortly after birth, and rapidly declines to undetectable levels, being replaced primarily with CYP3A4. Distinct isoform-specific developmental expression of CYPs has been noted postnatally in the following order of appearance: CYP2E1, CYP2D6, CYP3A4, CYP2C9/19, and CYP1A2 (180). CYP2E1 expression surges within hours after birth, CYP3A4 and CYP2C appear during the first week of life, and CYP1A2 appears at one to three months of life.

8. PHARMACOKINETIC/PHARMACODYNAMIC (PK/PD) MODELING OF PRECLINICAL DATA

PK/PD modeling was traditionally been utilized only during phase 2 to phase 4 trials, using clinical PK and efficacy data. Today, PK/PD modeling is also combined with computer simulations to design trials, prior to initiation of the clinical trials. Although the use of preclinical pharmacokinetic–pharmacodynamic modeling and simulation in drug development is presented in [Chapter 6](#), the topic will be briefly discussed here.

In recent years, modeling approaches have been applied to preclinical studies to predict first in human (FIH) or Proof of Concept (POF) dosing regimens. This approach is popularly becoming known as Computer Assisted Trial Design (CATD) or Computer Assisted Drug Development (CADD). Typically, for phase 2 to 4 trial designs, CATD simulates virtual clinical trials under various design scenarios to predict the outcomes of real-life clinical trials, based on mathematical models that reflect the following:

PK/PD relationship, distribution of patient characteristics, trial execution conditions (e.g., dropouts, noncompliance).

For designing FIH and/or POC clinical trials, CATD requires integration of toxicokinetic, pharmacokinetic, pharmacodynamic, and drug metabolism studies obtained from preclinical studies for identification of dosage regimens that result in optimal therapeutic outcome in POC studies prior to initiation of clinical full drug development. Simulation of a planned study allows one to fully evaluate and understand the consequences of several study design factors and assumptions before initiation of the actual clinical trial.

As discussed in detail in [Chapter 6](#), Gomeni et al. (182) recently published an article applying the CADD approach for FIH and POC dosing regimen predictions using preclinical data. A schematic flow diagram of the CADD approach implemented is shown in Fig. 1. A five-step approach was used: (I) allometric scaling of PK and protein binding data from rat, cynomolgus monkey, and dog; (II) PK/PD evaluation in rat; (III) simulations of rat PK/PD and scaled PK for rhesus monkey was used to predict PK/PD in rhesus monkey; (IV) predicted PD in rhesus monkey was compared to the observed PD in rhesus monkey in order to validate the model; (V) simulations of PK/PD in man from allometric scaling of PK and PD data.

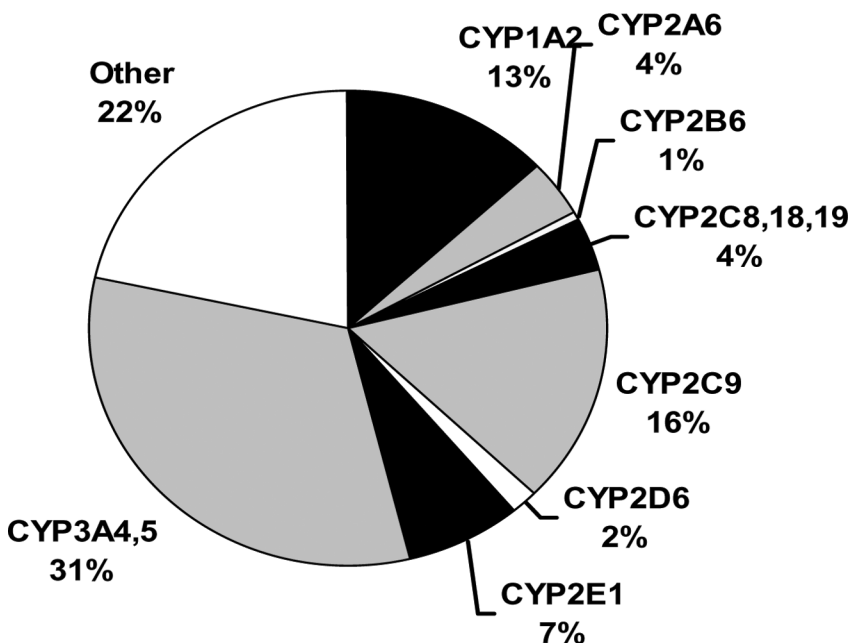


Figure 1 Relative distribution of drug metabolizing CYP isoforms in the human liver.

These approaches are relatively new and the success of these modeling and simulations for predicting outcome of FIH or POC studies requires additional investigations from pharmaceutical industry. Moreover, these approaches are time consuming and require integration of PK/PD data from both discovery and early development. A CATD scientist should initiate modeling and simulation analysis early in discovery when limited data are available and continuously update these models as more data are obtained through during preclinical development studies. This ensures that discovery and preclinical scientists collect the data required by the CATD scientist, and also safeguards against no loss of information during transition of drug development from discovery to early preclinical development to FIH and POC studies (Fig. 2).

9. CONCLUSIONS

It is clear that drug disposition is a very complex process and there is no unique mechanism that can result in an accurate prediction of human pharmacokinetic properties for every drug from preclinical studies. Nevertheless, the importance of the preclinical studies is to determine a safe dose for FIH clinical trials. In the future, preclinical data may be widely utilized to ensure for accurate design of Proof of Concept trials. To date, available models are empirical and extrapolation should be made very cautiously. However, continued retrospective and prospective analysis should make these models more accurate and predictive in nature. Future emphasis should be on combining animal PK/PD and TK/TD studies with in vitro animal and human studies. Where necessary application of physiologically based pharmacokinetic models can prove to be invaluable. In addition, exploratory research

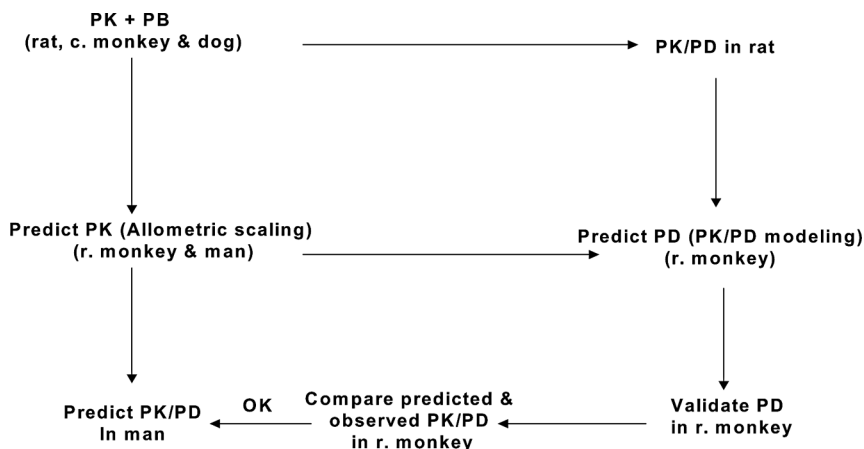


Figure 2 Preclinical PK/PD modeling approaches.

can be utilized to detect toxic mechanisms that are unrelated to pharmacokinetics, such as formation of reactive metabolite that are normally not detected in biofluids. The identification of the issues early during drug discovery and drug evaluation can help make informed go/no go decisions as well as ensure design of a well-defined clinical program. As a result, drug evaluation programs can be terminated at an early stage before futile investments are made or can be launched into clinical development with a high degree of success.

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Pharmacokinetics/ADME of Large Molecules

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1. INTRODUCTION

As discussed in previous chapters, pharmacokinetics (PK) is the study of the rate processes that are responsible for the time course of the level of an exogenous compound in the body. The processes involved are absorption (A), distribution (D), metabolism (M), and excretion (E). The pharmacokinetics of peptides, proteins, and other biotechnology products are an important factor in their pharmacodynamics, i.e., the time course of their pharmacological effect. Therefore, knowledge of the pharmacokinetics of a pharmaceutical drug in humans and laboratory animals is required when selecting dose levels and dose regimens. Similarly, the toxicokinetics (pharmacokinetics in toxicology studies, including higher doses than used clinically) are important for the design of toxicology studies (dose levels and dose regimens) as well as in determining safety margins and extrapolating toxicological data to humans.

In this chapter, the PK and ADME characteristics of protein therapeutics will be described. The ADME mechanisms for protein drugs that influence the plasma PK and systemic exposure are usually similar to those that handle endogenous proteins. Receptor-mediated uptake mechanisms that may also be involved in the protein's PD effect play an important role. This is generally different from small molecule drugs that are taken up by cells and distribute into organs, including the biophase, in many cases by

simple passive diffusion. In addition, receptor binding of small molecules is important for their PD effect but the fraction bound to the receptors rarely plays a role in their PK. Surprisingly, some large molecule drugs also bind to circulating plasma proteins, which may influence their PK and PD in similar ways as for small molecule drugs. Unlike small synthetic molecules for which different metabolic pathways exist in different species, the clearance mechanism for peptides and proteins is generally conserved across mammalian species. As a result, the PK of many protein drugs can be scaled mathematically between species. In contrast to most small molecule drugs, large molecule therapeutics may be immunogenic and circulating antibodies can influence their PK and PD.

2. CLEARANCE MECHANISMS OF PROTEIN THERAPEUTICS

It is commonly accepted that peptide and protein drugs are metabolized through identical catabolic pathways as endogenous and dietary proteins. Generally, proteins are broken down into amino acid fragments that can be re-utilized in the synthesis of endogenous proteins. Although history has shown that proteins can be powerful and potentially toxic compounds, their end products of metabolism are not considered to be a safety issue. This is in contrast with small organic synthetic drug molecules from which potentially toxic metabolites can be formed. The study of the metabolism of protein drugs is also very complicated because of the great number of fragments that can be produced. The mechanisms for elimination of peptides and proteins are outlined in [Table 1](#).

2.1. Proteolysis

Most, if not all, proteins are catabolized by proteolysis. Proteolytic enzymes are not only widespread throughout the body, they are also ubiquitous in nature, and therefore the potential number of catabolism sites on any protein is very large (1–3). It has been shown for interferon- α (IFN- α) that truncated forms are present in the circulation after dosing of rhesus monkeys with rIFN- α . The rate and extent of production of these metabolites may be dependent on the route of administration. This, and the cross-reactivity of these degraded forms in the ELISA may be responsible for the observation of a bioavailability of more than 100% after subcutaneous administration of rIFN- α (4). Proteolytic activity in tissue may be responsible for the loss of protein after subcutaneous administration.

2.2. Renal Excretion and Metabolism

Metabolism studies of peptide and protein drugs were performed to identify the organs responsible for metabolism (and/or excretion), and their relative contribution to the total elimination clearance. The importance of the

Table 1 Clearance Mechanisms for Peptides and Proteins as a Function of Molecular Weight (Mw). Other Determining Factors are Size, Charge, Lipophilicity, Functional Groups, Sugar Recognition, Vulnerability for Proteases, Aggregation to Particles, Formation of Complexes with Opsonization Factors, etc. The Indicated Mechanisms Overlap, and Fluid-phase Endocytosis can in Principle Occur Across the Entire Mw Range

Mw	Site of elimination	Dominating clearance mechanism	Determinant factor
<500	Blood	Extracellular hydrolysis	
	Liver	Passive nonionic diffusion	
500–1,000	Liver	Carrier-mediated uptake	Structure
		Passive nonionic diffusion	Lipophilicity
1,000–50,000	Kidney	Glomerular filtration	Mw
50,000–200,000	Kidney	Receptor-mediated endocytosis	Sugar, charge
	Liver	Receptor-mediated endocytosis	
200,000–400,000		Opsonization	α 2-macroglobulin IgG
			Particle aggregation
>400,000		Phagocytosis	

Source: From Ref. 21.

kidney as an organ of elimination was assessed for rIL-2 (5), M-CSF (6), and rIFN- α (7) in nephrectomized animals. The relative contributions of renal and hepatic clearances to the total plasma clearance of several other proteins are shown in Fig. 1.

The different renal processes that are important for the elimination of proteins are depicted in Fig. 2. The kidney appears to be the most dominant organ for the catabolism of small proteins (8). Based on the observation that only trace amounts of albumin pass the glomerulus, it is believed that macromolecules must be smaller than 69 kDa to undergo glomerular filtration (9). Glomerular filtration and excretion is most efficient for proteins smaller than 30 kDa (10). Peptides and small proteins (<5 kDa) are filtered very efficiently, and their glomerular filtration clearance approaches the glomerular filtration rate (GFR, $\sim$ 120 mL/min in humans). For molecular weights exceeding 30 kDa, the filtration rate falls off sharply. While there tends to be a reasonable correlation of clearance with molecular weight, the mechanism underlying this correlation is hydrodynamic volume. Indeed, it is the effective molecular radius that determines the degree of sieving by the glomerulus (Fig. 3) (11).

The glomerular barrier is also charge selective: the clearance of anionic molecules is impaired relative to that of neutral molecules, and the clearance

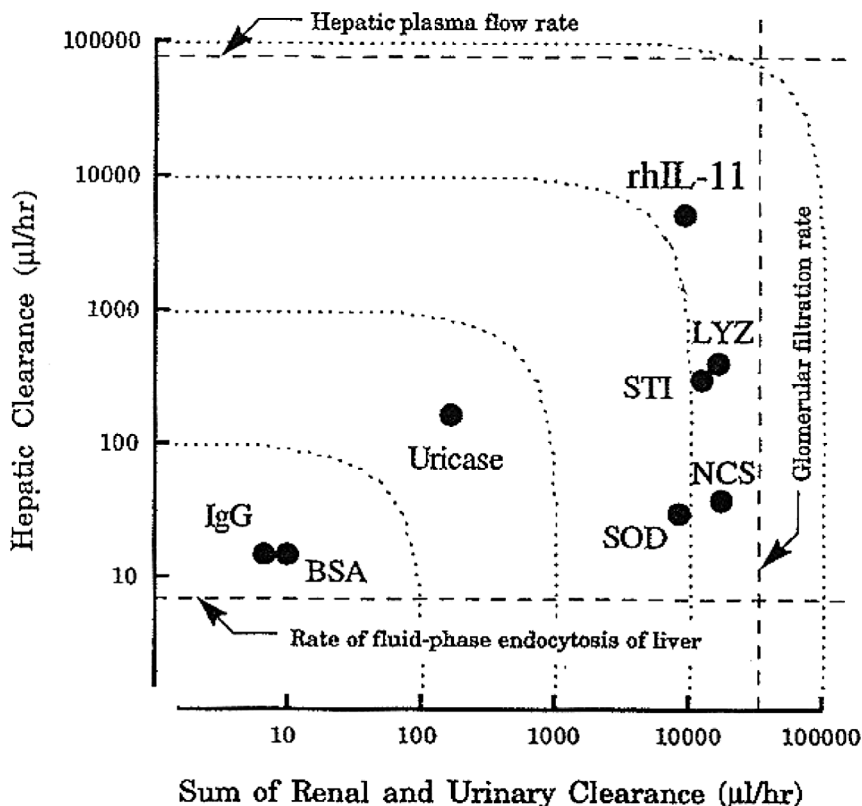


Figure 1 Hepatic and renal clearances of proteins in mice. LYZ, lysozyme; STI, soy bean trypsin inhibitor; NCS, neocarzinostatin; IgG, immunoglobulin G; BSA, bovine serum albumin and rhIL-11, recombinant human interleukin-11. (From Ref. 96.)

of cationic macromolecules is enhanced. The influence of charge on glomerular filtration is especially important for molecules with a radius greater than 2 nm (12). The charge-selectivity of glomerular filtration is related to the negative charge of the glomerular filter due to the abundance of glycosaminoglycans. Anionic proteins, such as TNF- α and INF- α , are therefore repelled (2).

After glomerular filtration, some peptides (melanostatin, for example) can be excreted unchanged in the urine. In contrast, more complex polypeptides and proteins are actively re-absorbed by the proximal tubules by luminal endocytosis and then hydrolyzed within the intracellular lysosomes to peptide fragments and amino acids (12,13). The amino acids are returned to the systemic circulation for reprocessing into new protein. Consequently,

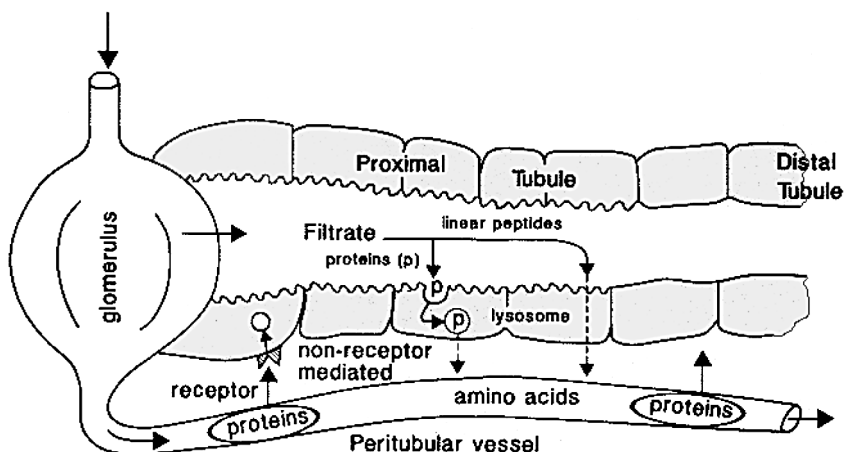


Figure 2 Pathways of renal elimination of proteins, including glomerular filtration, catabolism at the luminal membrane, tubular absorption followed by intracellular degradation, and postglomerular peritubular uptake followed by intracellular degradation. (From Ref. 11.)

only small amounts of intact protein are detected in the urine. The kidney appears to be the most dominant organ for the catabolism of small proteins (8). Examples of proteins undergoing tubular reabsorption are calcitonin, glucagons, insulin, growth hormone, oxytocin, vasopressin, and lysozyme (10). Cathepsin D, a major renal protease, is responsible for the hydrolysis of IL-2 in the kidney (14). Important determinants for tubular reabsorption of proteins are their physicochemical characteristics such as net charge and number of free amino groups (8). Cationic proteins are more susceptible to reabsorption than anionic proteins (15). Renal tubular cells also contain an active transporter for di- and tripeptides (16).

Small linear peptides (<10 amino acids) such as angiotensin I and II, bradykinin, and LHRH are subjected to luminal membrane hydrolysis. They are hydrolyzed by enzymes in the luminal surface of the brush border membrane of the proximal tubules, and the small peptide fragments and amino acids are subsequently reabsorbed, further degraded intracellularly, and/or transported through the cells into the systemic circulation (17).

Peritubular extraction of proteins from the postglomerular capillaries and intracellular catabolism is another renal mechanism of elimination (18). This route of elimination was demonstrated for IL-2 (5), insulin (19,20), calcitonin, parathyroid hormone, vasopressin, and angiotensin II (8). It is believed that the peritubular pathway exists mainly for the delivery of certain hormones to their site of action, i.e., to the receptors on the contraluminal site of the tubular cells.

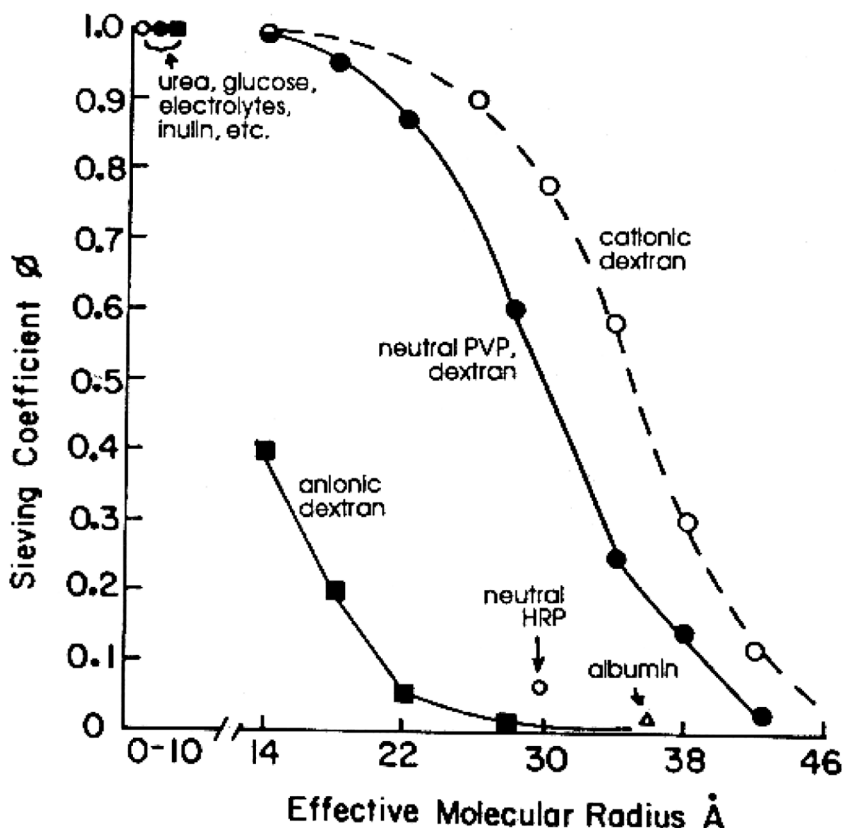


Figure 3 Sieving curves of several macromolecules. The different sieving coefficients reflect the influence of size, charge, and rigidity of molecules. (From Ref. 97.)

2.3. Hepatic Metabolism

Besides proteolytic enzymes and renal catabolism, the liver has also been shown to contribute significantly to the metabolism of protein therapeutics. The rate of hepatic catabolism, which determines in part the elimination half-life, is largely dependent on the presence of specific amino acid sequences in the protein (21). Before intracellular hepatic catabolism, proteins and peptides need to be transported from the blood stream to the liver cells. An overview of the different mechanisms of hepatic uptake of proteins is listed in [Table 2](#).

Molecules of relatively small size and with highly hydrophobic characteristics permeate the hepatocyte membrane by simple nonionic passive diffusion. Peptides of this nature include the cyclosporins (cyclic peptides) (22). Other cyclic and linear peptides of small size (<1.4 kDa) and hydrophobic

nature (containing aromatic amino acids), such as renin and cholecystokinin-8 (CCK-8; 8 amino acids), are cleared by the hepatocytes by a carrier-mediated transport (22). After internalization into the cytosol, these peptides are commonly metabolized by microsomal enzymes (cytochrome P-450III_A for cyclosporin A) or cytosolic peptidases (CCK-8). Substances that enter the liver via carrier-mediated transport are typically excreted into the bile by the multispecific bile-acid transporter. These hepatic clearance pathways are identical to those known for most small organic hydrophobic drug molecules.

For larger peptides and proteins, there is a multitude of energy-dependent carrier-mediated transport processes available for cellular uptake. One of the possibilities is receptor-mediated endocytosis (RME), such as for insulin and epidermal growth factor (EGF) (23–25). In RME, circulating proteins are recognized by specific hepatic receptor proteins (10). The receptors are usually integral membrane glycoproteins with an exposed binding domain on the extracellular side of the cell membrane. After binding of the circulating protein to the receptor, the complex is already present or moves in coated pit regions, and the membrane invaginates and pinches off to form an endocytotic coated vehicle that contains the receptor and ligand (internalization). The vesicle coat consists of proteins (clathrin, adaptin, and others), that are then removed by an uncoating adenosine triphosphatase (ATPase). The vesicle parts, the receptor, and the ligand dissociate and are targeted to various intracellular locations. Some receptors, such as the LDL, asialoglycoprotein and transferrin receptors, are known to undergo recycling. Since sometimes several hundred cycles are part of a single receptor's lifetime, the associated RME is of high capacity. Other receptors, such as the interferon receptor, undergo degradation. This leads to a decrease in the concentration of receptors on the cell surface (receptor down-regulation). Others (insulin and EGF receptors, for example) undergo both recycling and degradation (10).

For glycoproteins, if a critical number of exposed sugar groups (mannose, galactose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, or glucose) are exceeded, RME through sugar-recognizing receptors is an efficient hepatic uptake mechanism (21). Important carbohydrate receptors in the liver are the asialoglycoprotein receptor in hepatocytes and the mannose receptor in Kupffer and liver endothelial cells (26–28). The high-mannose glycans in the first kringle domain of rt-PA have been implicated in its clearance, for example (29).

Low density lipoprotein receptor-related protein (LRP) is a member of the low-density lipoprotein (LDL) receptor family responsible for endocytosis of several important lipoproteins, proteases, and protease-inhibitor complexes in the liver and other tissues (30). Examples of proteins and protein complexes for which hepatic uptake is mediated by LRP are listed in [Table 2](#). The list includes many endogenous proteins, including some that are

Table 2 Hepatic Uptake Mechanisms for Proteins and Protein Complexes

Cell type/organ	Uptake mechanism	Proteins/peptides transported
Hepatocytes	Anionic passive diffusion	Cyclic and linear hydrophobic peptides (<1.4 kDa) (cyclosporins, CCK-8)
	Carrier-mediated transport	
	RME: Gal/GalNAc receptor (Asialoglycoprotein receptor)	<i>N</i> -acetylgalactosamine-terminated glycoproteins Galactose-terminated glycoproteins (e.g., desialylated EPO)
	RME: low density lipoprotein receptor (LDLR)	LDL, apoE- and apoB-containing lipoproteins
	RME: LDLR-related protein (LRP receptor)	α_2 macroglobulin, apoE-enriched lipoproteins, Lipoprotein lipase, (LpL), Lactoferrin, t-PA, u-PA, Complexes of t-PA and u-PA with plasminogen activator inhibitor type 1 (PAI-1), TFPI, Thrombospondin (TSP), TGF- β and IL-1 β bound to α_2 -macroglobulin
	RME: other receptors	IgA, Glycoproteins, Lipoproteins, Immunoglobulins, Intestinal and pancreatic peptides, Metallo- and hemoproteins, Transferrin, Insulin, Glucagon, GH, EGF
	Nonselective pinocytosis (nonreceptor-mediated)	Albumin, Antigen-antibody complexes, Some pancreatic proteins, Some glycoproteins
Kupffer cells	Endocytosis	Particulates with galactose groups

Kupffer and endothelial cells	RME	IgG-type antibodies
		<i>N</i> -acetylglucosamine-terminated glycoproteins
	RME: mannose receptor	Mannose-terminated glycoproteins (e.g., t-PA, renin)
Endothelial cells	RME: fucose receptor	Fucose-terminated glycoproteins
	RME: scavenger receptor	Negatively charged proteins
	Pinocytosis + binding to the Fc receptor (Brambell or FcRN salvage receptor) and recycling	IgG-type antibodies
Fat-storing cells	RME: other receptors	VEGF, FGF (?)
	RME: mannose-6-phosphate receptor	Mannose-6-phosphate-terminated proteins (e.g., IGF-II)
Liver, spleen	Fixed tissue macrophages	Immune complexes (antigen–antibody complexes)

Source: From Refs. 99, compiled from several sources (see references in the text), including reviews in [Refs. 10, 100, 29](#).

RME = Receptor-mediated endocytosis.

marketed or being developed as drugs, such as t-PA, u-PA, and tissue factor pathway inhibitor (TFPI). There are observations indicating that these proteins bound to the cell-surface proteoglycans are presented to LRP for endocytosis, thus facilitating the LRP-mediated clearance. It seems likely that proteoglycans serve to concentrate LRP ligands on the cell surface, thereby enhancing their interaction with LRP. Interestingly, none of the LRP ligands compete against each other for the LRP receptor, which is very large (~650 kDa) and contains multiple distinct binding sites (31).

Uptake of proteins by liver cells is followed by transport to an intracellular compartment for metabolism. Proteins internalized into vesicles via an endocytotic mechanism such as RME undergo intracellular transport towards the lysosomal compartment near the center of the cell. There, the endocytotic vehicles fuse with or mature into lysosomes, which are specialized acidic vesicles that contain a wide variety of hydrolases capable of degrading all biological macromolecules. Proteolysis is started by endopeptidases (mainly cathepsin D) that act on the middle part of the proteins. The resulting oligopeptide metabolites are further degraded by exopeptidases. The final metabolic products, amino acids, and dipeptides, reenter the metabolic pool of the cell (21). The hepatic metabolism of glycoproteins may occur slower than the naked protein because protecting oligosaccharide chains must be removed prior to hydrolysis of the amino acid backbone. Metabolized proteins and peptides in lysosomes from hepatocytes, hepatic sinusoidal cells and Kupffer cells may be released into the blood. Degraded proteins in hepatocyte lysosomes can also be delivered to the bile canaliculus and excreted by exocytosis.

A second intracellular clearance pathway for proteins is the direct shuttle or transcytotic pathway (10). The endocytotic vesicle formed at the cell surface traverses the cell to the peribiliary space, where it fuses with the bile canalicular membrane, releasing its contents by exocytosis into bile. This pathway described for polymeric immunoglobulin A, bypasses the lysosomal compartment completely.

Receptor-mediated uptake of protein drugs by hepatocytes, followed by intracellular metabolism, may cause dose-dependent plasma disposition curves due to the saturation of the active uptake mechanism at higher doses. As an example, EGF administered at low doses (50 $\mu\text{g/kg}$ and lower) to rats showed an elimination clearance proportional to hepatic blood flow, since the systemic supply of drug to the liver is the rate limiting process for elimination. At high doses (>200 $\mu\text{g/kg}$), the hepatic clearance is saturated, and extrahepatic clearance by other tissues becomes the dominant factor in the total plasma clearance. At intermediate doses of EGF, both hepatic blood flow and EGF receptors responsible for the active uptake affect the total plasma clearance (32).

For some proteins, receptor-mediated uptake by the hepatocytes is so extensive that hepatic blood clearance approaches its maximum value, the

liver blood flow. As examples, recombinant tissue-type and urokinase-type plasminogen activator (rt-PA and ru-PA, respectively) have been shown to behave as high clearance drugs, and both reductions and increases in liver blood flow affect their clearance in the same direction (33,34). This physiological parameter may have therapeutic implications in patients with myocardial infarction since they can experience variations in liver perfusion caused by diminished cardiac function or concomitant vasoactive drug treatment. Also, liver blood flow decreases during exercise, and increases after food intake.

2.4. Receptor-Mediated Elimination by Other Cells

For small synthetic drugs, the fraction of the dose bound to receptors at any moment after administration is usually negligible, and receptor binding is reversible, mostly without internalization of the receptor–drug complex. For protein drugs, however, a substantial part of the dose may be bound to the receptor, and receptor-mediated uptake by specialized cells followed by intracellular catabolism may play an important part in the total elimination of the drug from the body. A derivative of granulocyte colony-stimulating factor (G-CSF), nartogristim, and most likely G-CSF itself, is taken up by bone marrow through a saturable receptor-mediated process (35). It has been demonstrated for macrophage colony-stimulating factor (M-CSF) that besides the linear renal elimination pathway, there is a saturable nonlinear elimination pathway that follows Michaelis–Menten kinetics (6,36). The importance of the nonlinear elimination pathway was demonstrated by a steeper dip in the plasma concentration profile at lower M-CSF concentrations (Fig. 4). At higher levels, linear renal elimination was dominant, and the nonlinear pathway was saturated. The nonlinear pathway could be blocked by coadministration of carrageenan, a macrophage inhibitor, indicating that receptor-mediated uptake by macrophages was likely responsible for the nonlinear elimination (6). This is especially relevant since M-CSF stimulates the proliferation of macrophages. It is also possible that the receptor-mediated uptake and the effect of M-CSF are closely linked. Indeed, it was observed that after chronic administration of M-CSF, the nonlinear elimination was probably induced by autoinduction since M-CSF increases circulating levels of macrophages. Although autoinduction and consequently accelerated metabolism of most drugs is related to a loss of their pharmacological effect, for M-CSF, it may be an indication of sustained pharmacodynamic activity. Similar kinetics was observed for other hematopoietic stimulating factors such as G-CSF (37) and GM-CSF (38). Michaelis–Menten (saturable) elimination was also described for t-PA (39), and recently for a recombinant amino-terminal fragment of bactericidal/permeability-increasing protein (rBPI₂₃) (40).

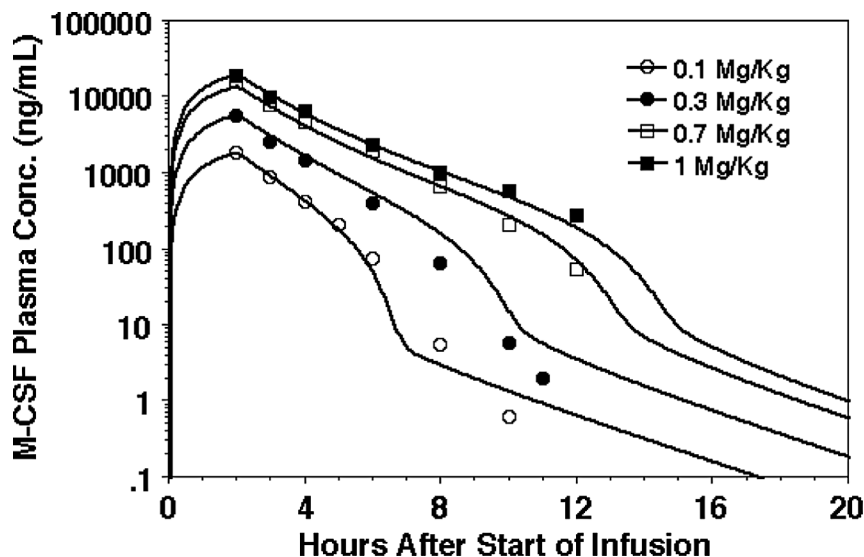


Figure 4 Observed and predicted plasma concentration–time profiles of M-CSF after 2-hr intravenous infusions of 0.1–1 mg/kg in cynomolgus monkeys. A two-compartmental pharmacokinetic model with a linear clearance pathway and a parallel Michaelis–Menten elimination pathway was used.

In recent years, several monoclonal antibodies have reached the market, and currently nearly 25% of pharmaceutical biotech products in development are believed to be antibodies or antibody derivatives (41). Their unique structure results in interesting pharmacokinetics. The Fc domain of antibodies and their size is largely responsible for their pharmacokinetic properties with systemic half-lives of several days to weeks. The large size (>150 kDa) prevents excretion through the kidneys. Resistance to proteases is another reason for long half-lives. IgG-type antibodies have an additional mechanism that contributes to their very long half-lives (1–3 weeks). Endothelial cells take up most serum proteins by pinocytosis and are consequently degraded in the endothelial cells. IgG antibodies however contain a region in their Fc domain that is recognized by the Fc receptor, called the Brambell receptor, FcRN or salvage receptor. When IgG molecules enter the endothelium, they bind to this receptor in the endosomal compartment, after which the complex moves to the cell surface and the IgG molecule is again liberated into the circulation. This recycling mechanism accounts for the long half-life of IgG-type antibodies (41). Immune complexes (antibodies bound to antigen) are transported to the liver and spleen where they are taken up and degraded by tissue macrophages. This pathway, which is also responsible for the clearance of colloidal particulates (<5 μm) and classical liposomes, is termed the mononuclear phagocyte system (MPS).

3. DISTRIBUTION OF PROTEIN THERAPEUTICS

Once a molecule reaches the blood stream, it encounters the following processes for intracellular biodistribution: distribution within the vascular space, transport across the microvascular wall, transport through the interstitial space, and transport across cell membranes. The biodistribution of macromolecules is determined by the physicochemical properties of the molecule, and by the structural and physicochemical characteristics of the capillaries responsible for transendothelial passage of the molecule from the systemic circulation to the interstitial fluid. In addition, the presence of receptors determines the biodistribution to certain tissues, including extracellular association and/or intracellular uptake. Capillary endothelia are of three types, in increasing order of permeability: continuous (nonfenestrated), fenestrated, and discontinuous (sinusoidal) (10,42). The most likely dominant mode of transport of macromolecules in nonfenestrated capillaries is through interendothelial junctions. Through these junctions, there are two modes of transport (43): the convective transport, often the most important for macromolecules, is dependent on a pressure difference between the vascular and interstitial spaces and the diffusive transport is driven by a concentration gradient.

Capillaries selectively sieve macromolecules based on their effective molecular size, shape, and charge. Because of the large size of proteins, their apparent volume of distribution is usually relatively small. The initial volume of distribution after intravenous injection is approximately equal to or slightly higher than the total plasma volume. The total volume of distribution is generally up to two times the initial volume of distribution. Although this is sometimes interpreted as a low tissue penetration, it is difficult to generalize. Indeed, adequate concentrations may be reached in a single target organ because of receptor-mediated uptake, but the contribution to the total volume of distribution may be rather small.

In addition to size, it appears that the charge-selective nature of continuous capillaries and cell membranes may also be important for the biodistribution of proteins. Information for this is available from studies with different types of Cu, Zn-superoxide dismutase (Cu, Zn-SOD), which are similar in molecular weight (33 kDa), but have different net surface charges, and are isolated from different species (44). Tissue equilibration of the positively charged sheep Cu, Zn-SOD was much faster than for the negatively charged bovine Cu, Zn-SOD. In addition, the positively charged Mn-SOD equilibrated much faster than the negatively charged human Cu, Zn-SOD, although Mn-SOD is much bigger (88 kDa). A trend towards increasing anti-inflammatory activity, for which interstitial concentrations are important, was observed with increasing isoelectric point. It was suggested that the electrostatic attraction between positively charged proteins and negatively charged cell membranes might increase the rate and extend of tissue

biodistribution. Most cell surfaces are negatively charged because of the abundance of glycosaminoglycans in the extracellular matrix.

Tissue binding is also important for the biodistribution of the heparin-binding proteins, including the fibroblast growth factor family (such as FGF-1 and FGF-2) (45), vascular endothelial growth factor (VEGF) (46), platelet-derived growth factor (PDGF), TFPI (47), amphiregulin (AR), and EGF. Proteins of this group contain a highly positively charged tail, which electrostatically binds to low-affinity binding sites consisting of heparin sulfate proteoglycans (acidic glycosaminoglycans) (48,49). These binding sites are abundant on the vascular endothelium and liver, and are responsible for the majority of cell surface binding of these proteins. The rapid and extensive binding to the vascular endothelium of protein drugs in this class is most likely the explanation for their rapid distribution phase after i.v. injection, and their relatively large volume of distribution. Binding of growth factors to proteoglycans has been proposed to provide a mechanism for growth factor recruitment at the cell surface, presentation to specific receptors, regulation of their action on target cells at short range, and establishment of a growth factor gradient within a tissue.

A major *in vivo* pool of some of the heparin-binding proteins appears to be associated with the vascular endothelium, and is released into the circulation quickly after injection of heparin. Since heparin is structurally similar to the cell-surface glycosaminoglycans, the proteins bind to circulating heparin, depleting the intravascular pool. This was demonstrated, for example, for TFPI (50,51) and basic FGF (FGF-2) (45).

Biodistribution studies with the measurement of the protein drug in tissues are necessary to establish tissue distribution. These studies are usually performed with radiolabeled compounds. Biodistribution studies are imperative for small organic synthetic drugs since long residence times of the radioactive label in certain tissues may be an indication of tissue accumulation of potentially toxic metabolites. Because of the possibility of reutilization of amino acids from protein drugs in endogenous proteins, such a safety issue does not exist. Therefore, biodistribution studies for protein drugs are usually performed to assist drug targeting to specific tissues, or to detect the major organs of elimination (usually kidneys and liver).

If the protein contains a suitable amino acid such as tyrosine or lysine, an external label such as ^{125}I can be chemically coupled to the protein (4). Although this is easily accomplished and a high specific activity can be obtained, the protein is chemically altered. Therefore, it may be better to label proteins and other biotechnology compounds by introducing radioactive isotopes during their synthesis by which an internal atom becomes the radioactive marker (internal labeling). For recombinant proteins, this can be accomplished by growing the production cell line in the presence of amino acids labeled with ^3H , ^{14}C , ^{35}S , etc. This method is not routinely used because of the prohibition of radioactive contamination of fermentation equipment.

Moreover, internally labeled proteins may be less desirable than iodinated proteins because of the potential for re-utilization of the radiolabeled amino acid fragments in the synthesis of endogenous proteins and cell structures. Irrespective of the labeling method, the labeled product should demonstrate physicochemical and biological properties identical to the unlabeled molecule (52).

In addition, as for all types of radiolabeled studies, it needs to be established whether the measured radioactivity represents intact labeled protein, or radiolabeled metabolites, or the liberated label. Trichloro-acetic acid-precipitable radioactivity is often used to distinguish intact protein from free label or low-molecular-weight metabolites, which appear in the supernatant after centrifugation. Proteins with re-utilized labeled amino acids and large protein metabolites can only be distinguished from the original protein by techniques such as PAGE, HPLC, specific immunoassays, or bioassays. This discussion implies also that the results of biodistribution studies with autoradiography can be very misleading. Although autoradiography is becoming more quantitative, one does not know what is being measured qualitatively without specific assays. It is therefore, sometimes better to perform biodistribution studies by collection of the tissues, and use specific measurement of the protein drug in the tissue homogenate.

A method was developed to calculate early-phase tissue uptake clearances based on plasma and tissue drug measurements during the first 5 min after intravenous administration (25). The short time interval has the advantage that metabolism and the tissue efflux clearance presumably can be ignored. As an example, with this method, dose-independent (nonsaturable) uptake clearance values were observed for a recombinant derivative of hG-CSF, nartograstim, for kidney and liver (35). In contrast, a dose-dependent reduction in the uptake clearance by bone marrow with increasing doses of nartograstim was observed. These findings suggested that receptor mediated endocytosis of the G-CSF receptor in bone marrow may participate in the nonlinear properties of nartograstim. Since G-CSF is one of the growth factors that stimulates the proliferation and differentiation of neutropoietic progenitor cells to granulocytes in bone marrow, the distribution aspects of nartograstim into bone marrow are especially relevant for the pharmacodynamics. In addition, since G-CSF and nartograstim are catabolized in the bone marrow cells after receptor-mediated uptake, the biodistribution into bone marrow is also a pathway for elimination of these molecules. Unlike for classical small synthetic drugs, it is not uncommon for biotechnology-derived drugs that biodistribution, pharmacodynamics, and elimination are closely connected.

Besides receptor-mediated uptake into target organs and tissues, other proteins, or macromolecules in general, distribute into tissues in more non-specific ways. It was demonstrated in at least one study with tumor-bearing mice that high total systemic exposure of target-nonspecific macromolecules

was the most important factor that determines the extent of tissue uptake (9). Consequently, molecules with physicochemical characteristics that minimize hepatic and renal elimination clearances showed the highest tumoral exposure. Compounds with relatively low molecular weights (approximately 10 kDa) or positive charges were rapidly eliminated and showed lower tumor radioactivity accumulation; large (>70 kDa) and negatively charged compounds (carboxymethyl dextran, BSA, mouse IgG) showed prolonged retention in the circulation, and high tumoral levels. A typical example is the murine urokinase (muPA) EGF-like domain peptide of 48 amino acids, muPA(1–48). This peptide is a urokinase receptor antagonist under consideration as an anticancer drug since urokinase has been implicated in invasive biological processes such as tumor metastasis, trophoblast implantation, and angiogenesis. Scientists at Chiron have fused muPA(1–48) to the human IgG constant region. The fused molecule (IgG–muPA(1–48)) retained its activity of inhibition of the murine UPA receptor, but has a much longer in vivo elimination half-life (79 vs. 0.5 hr; Fig. 5). The half-life increase was due to both a decrease in elimination clearance (4.3 vs. 95 mL/hr/kg) and an increase in the peripheral volume of distribution (434 vs. 43 mL/kg). Although the fused molecule was substantially larger, tissue distribution increased, possibly because of substantial tissue binding. This is in contrast

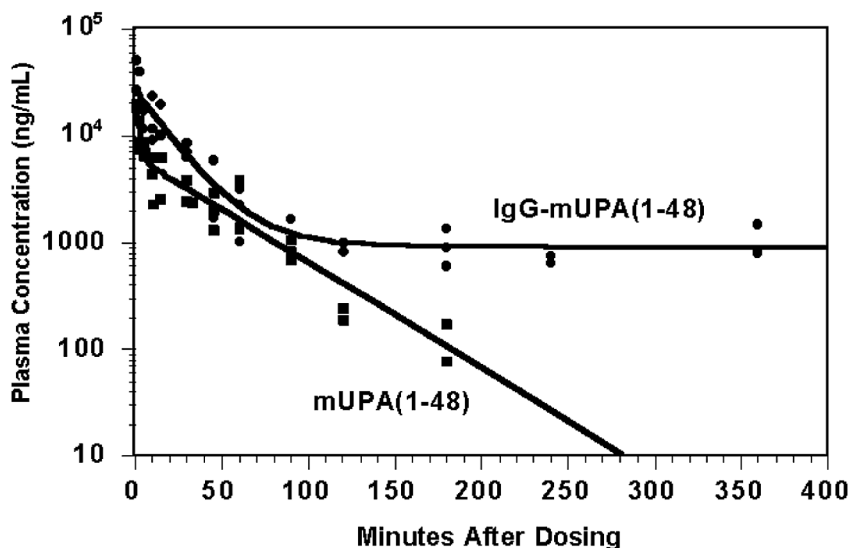


Figure 5 Fusion of the murine urokinase EGF-like peptide of 48 amino acids with human IgG (IgG–mUPA(1–48)) resulted in a much longer half-life than the original peptide (mUPA(1–48)). The data were modeled according to a linear two-compartmental model.

with some polyethylene glycol-modified (PEGylated) molecules such as PEG IL-2 (polyethylene glycol-modified interleukin-2) for which the size increase resulted in a smaller distribution volume compared to the original molecule (see below).

Biodistribution into the lymphatics after subcutaneous (s.c.) injection deserves special attention since it is a rather unique transport pathway for macromolecules. Following s.c. administration, the drug can be transported to the systemic circulation by absorption into the blood capillaries or by the lymphatics. Since the permeability of macromolecules through the capillary wall is low, they were found to enter blood indirectly through the lymphatic system (53,54). Compounds with a molecular weight larger than 16 kDa are absorbed mainly (>50%) by the lymphatics, while compounds smaller than 1 kDa are hardly absorbed by the lymphatics at all. Lymph recovery after s.c. dosing was apparently linearly related to molecular weight up to 19 kDa (Fig. 6) (54). Negatively charged proteins had increased lymph absorption as compared to positively charged proteins with similar

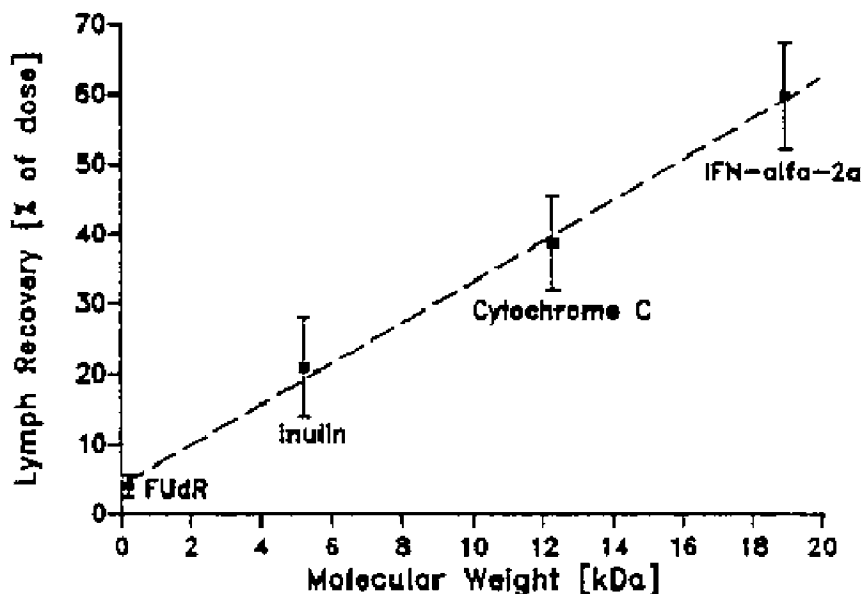


Figure 6 Correlation between the molecular weight and cumulative recovery of rIFN alpha-2a (Mw 19 kDa), cytochrome c (Mw 12.3 kDa), inulin (Mw 5.2 kDa), and FUDR (Mw 256.2 kDa) in the efferent lymph from the right popliteal lymph node following s.c. administration into the lower part of the hind leg of sheep. Each point and bar show the mean and standard deviation of 3 experiments performed in different sheep. The line drawn is the best fit by linear regression analysis calculated with the 4 mean values (correlation coefficient r of 0.998, $p < 0.01$). (From Ref. 54.)

molecular weight (55). After lymphatic absorption, compounds circulate within the lymph and are gradually returned to the blood. As a result, lymph concentrations for these proteins may be higher than blood concentrations. Targeting of the lymphatics may be beneficial for proteins that act on the immune system, such as for IL-2. It was shown that s.c. administration of IL-2 in a pig model resulted in higher lymph levels as compared to blood, and at higher doses, absorption was exclusively through lymph (56). The IL-2 receptor-positive T-lymphocytes, that are thought to be primarily associated with efficacy, reside largely in the lymphoid organs. On the other hand, natural killer cells and neutrophils in blood produce cytokines, reactive oxygen intermediates, and proteases, all of which have been shown to be necessary to produce IL-2 toxicities. Therefore, adverse *in vivo* activity of IL-2 may be related to blood levels, while beneficial activity may be associated to lymph concentrations (56).

Biodistribution into target organs, usually receptor-mediated, is important for the pharmacodynamics of protein drugs. For some proteins, saturable receptor-mediated tissue uptake in target organs is responsible for nonlinear kinetics (57). For example, the uptake clearance of rhEPO by bone marrow and spleen exhibited clear saturation in rats. Also, a single high dose of rhEPO caused a reduction of uptake clearance by bone marrow and spleen, while repeated injections caused an increase of the tissue uptake clearance, especially by the spleen, in a dose-dependent manner (57). Hematopoietic parameters such as hematocrit and hemoglobin concentration changed accordingly, suggesting that changes in the uptake clearance were caused by down- or upregulation of EPO receptors.

4. PLASMA PHARMACOKINETICS

Although the time course of the compound at the receptor or effector site is the desired knowledge to predict or explain the pharmacodynamics (PD), accurate drug level data at that site are difficult to obtain. In most cases, pharmacokinetic (PK) data are limited to plasma concentration data. Pharmacokinetic models are widely used to describe and predict the time course of the drug in plasma and tissues. These models include compartmental models and physiological models. A scan of the literature shows that mostly compartmental models are used, in particular one- or two-compartmental models. Terminal-phase elimination half-lives for small to medium sized protein drugs in humans range from a couple of hours (e.g., 3.7 hr for rt-PA) to more than 12 hr (e.g., 15 hr for Factor VIII). Very large protein drugs such as monoclonal antibody-based pharmaceuticals have plasma half-lives ranging from days to several weeks. The IgG1-based recombinant humanized monoclonal antibody trastuzumab (Herceptin α , 148 kDa) for example has a half-life that ranges from 1.7 days to 15 days after *i.v.* doses of 10 and 500 mg, respectively (58).

As a modeling example, the PK/PD model used for insulin after a single 10-U subcutaneous dose in 10 volunteers is depicted in Fig. 7 (59,60). The PK model consisted of a classical two-compartment model with first-order elimination from the central compartment. A hypothetical effect compartment is linked to the central compartment to model the PD of insulin, which in this case was the glucose infusion rate to maintain euglycemia. Figure 8 shows the mean serum concentration profile of insulin after a single s.c. injection of 10 U in 10 volunteers, and the corresponding effect measured as the glucose infusion rate to maintain an euglycemic state. The concentration in the effect compartment drives the intensity of the pharmacodynamic effect: the effect compartment of this PK/PD link model cannot be distinguished from the other compartments based on plasma concentration only. Compartmental modeling with plasma concentration-time data is in most cases just not sensitive enough to isolate the biophase as a separate compartment without the availability of measured drug concentration data in the biophase. Drug distributes into the effect compartment but since the amount of drug in the effect compartment is rather small, no actual mass transfer is implemented in the pharmacokinetic part of the PK/PD model.

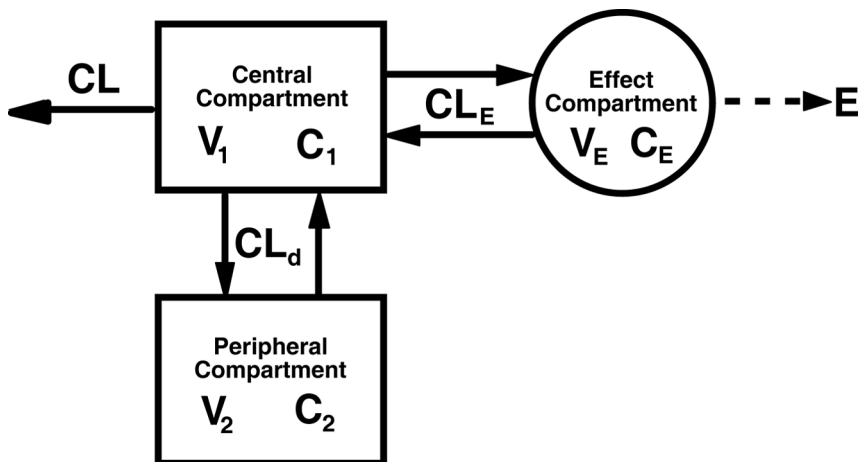


Figure 7 Example of a typical PK/PD link model (98). The PK model is a two-compartmental model with a linear elimination clearance from the central compartment (CL) and a distributional clearance (CL_d). C_1 and C_2 are the concentrations in the central and peripheral compartments, and V_1 and V_2 are their respective apparent volumes of distribution. A hypothetical effect compartment is linked to the central compartment. The concentration in the effect compartment (C_E) drives the intensity of the pharmacodynamic effect (E). CL_E is the linear clearance for distribution of drug to the effect compartment and elimination from the effect compartment. V_E is the apparent volume of distribution in the effect compartment.

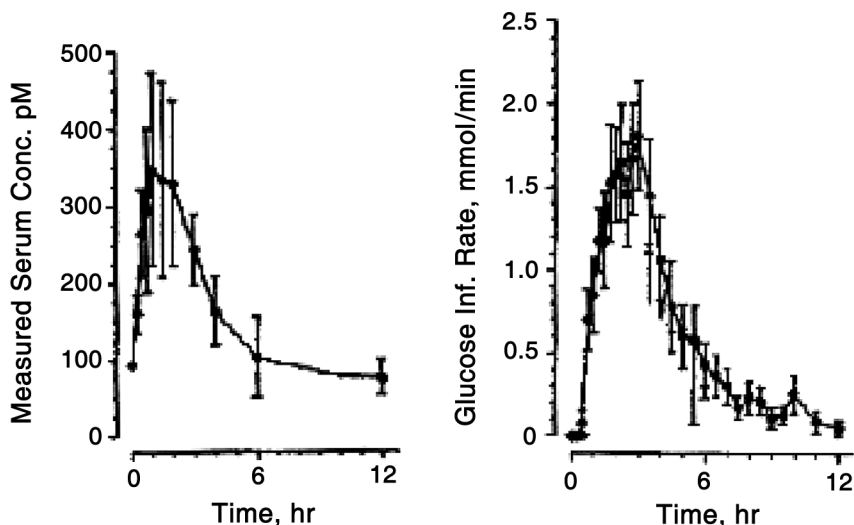


Figure 8 Mean measured serum insulin concentrations after a single 10-U subcutaneous dose of regular insulin in 10 volunteers (left panel); corresponding glucose infusion rates needed to maintain euglycemia (right panel). (From Ref. 60.)

This PK/PD link model accounts for the temporal delay of the effect appearance. The delay is typically explained by a distributional delay (61): drug concentrations in a slowly equilibrating tissue compartment with plasma are directly related to the effect intensity. Since the peak level of drug in the biophase is reached later than the time of the peak plasma concentration, the peak effect also occurs later than the plasma peak level. Although this PK/PD model is constructed with tissue distribution as the reason for the delay of the effect, the distribution clearance to the effect compartment can be interpreted differently, including other reasons of delay, such as transduction processes and secondary post receptor events.

4.1. Nonlinear Plasma Pharmacokinetics

As described earlier, many protein drugs are eliminated by receptor-mediated uptake in the liver or by other cells. Sometimes, this uptake is saturated at higher doses leading to dose-related nonlinearity whereby an increase in dose size does not result in a proportional increase in systemic drug exposure. In other instances, the receptors are upregulated after chronic exposure leading to time-related nonlinearity whereby the same dose at a later time after chronic dosing causes a lower drug exposure than after the first dose. As an example, the serum pharmacokinetics of filgrastim (r-methionine-hG-CSF) after s.c. dosing of human volunteers for 10 days

and the effect on neutrophilic granulopoiesis after s.c. dosing of human volunteers for 10 days (62) is shown in Fig. 9. The pharmacokinetics were modeled with a two-compartment model with elimination from the central compartment. The filgrastim clearance increases because of a time-related increase of the uptake receptors on the neutrophils or because of an increase in the total neutrophil count. This accounts for the observation that the filgrastim peak levels decrease as a function of time (Fig. 10). However, despite decreasing serum levels of filgrastim with chronic dosing, the pharmacodynamic effect increases and approaches a steady state after approximately six days. The indirect-effect PD model used to model the absolute neutrophil count (ANC) is shown in Fig. 9. The transient decrease of blood neutrophils in the first hour after dosing is due to rapid distribution of neutrophils into the marginal blood pool (disappearance process in Fig. 10). The increase in neutrophil count is modeled as a filgrastim concentration-dependent flux into the circulating neutrophil pool (appearance process in Fig. 10). This combined PK/PD model accurately describes the accession of the ANC to steady-state levels (Fig. 9). This example shows how multiple dose PK/PD data from human trials with nonlinearity in the PK and indirect PD effects can be modeled and predicted.

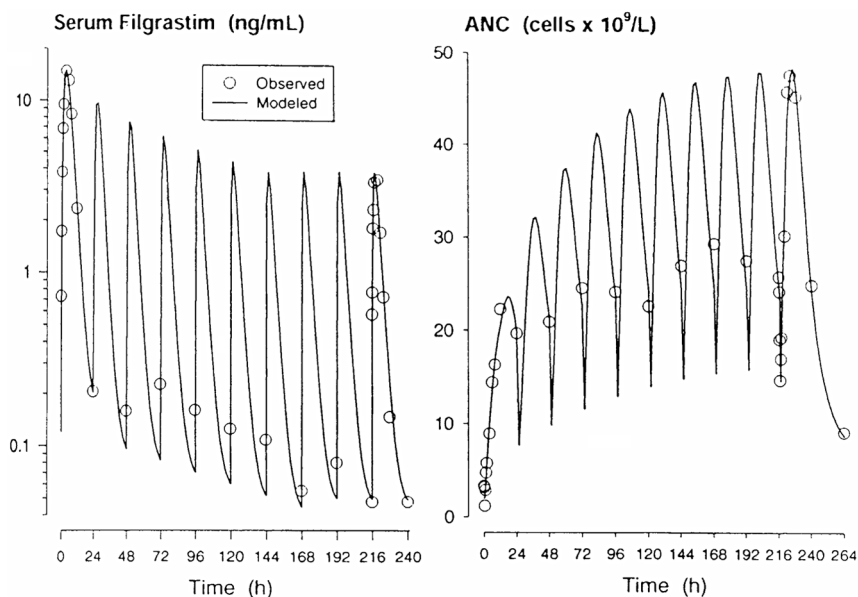


Figure 9 Simultaneous PK/PD modeling of serum filgrastim levels and mean absolute neutrophil counts (ANC) response in normal volunteers receiving s.c. filgrastim (300 µg/day) for 10 days (62).

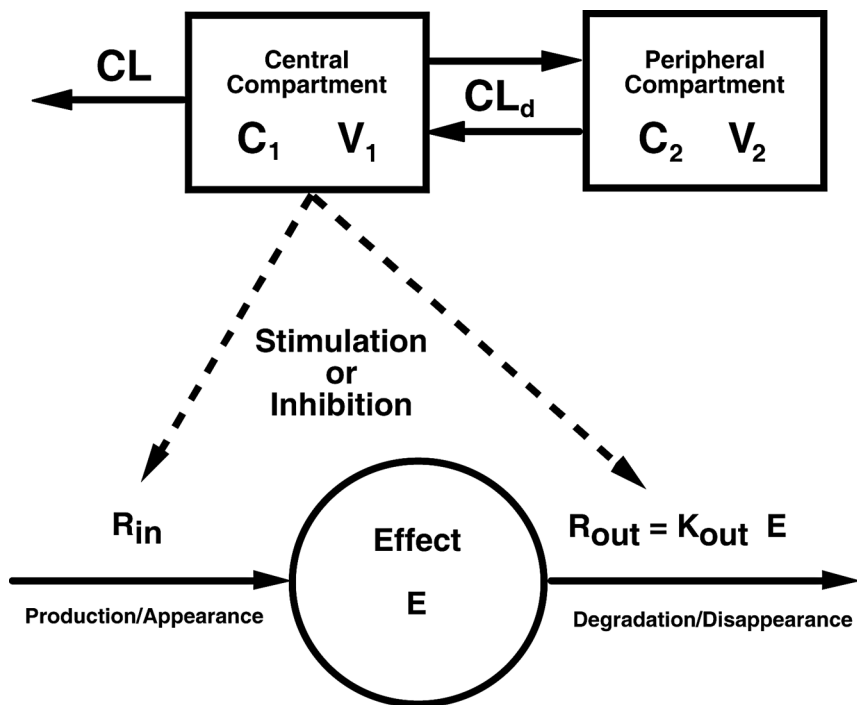


Figure 10 Pharmacodynamic indirect effect model wherein the effect is maintained by equilibrium between a zero-order appearance rate, R_{in} , and a first-order disappearance rate, R_{out} . A drug effect is caused by stimulation or inhibition of R_{in} or R_{out} . The degree of stimulation or inhibition is dependent on the plasma drug concentration. The PD parameters are R_{in} , K_{out} (the first order rate constant for effect disappearance), EC_{50} (the concentration that produces 50% of maximum inhibition or stimulation), and E_{max} (the maximum inhibition or stimulation). The pharmacokinetic model is identical as in Fig. 7. For filgrastim (see Fig. 9), R_{out} is transiently stimulated in the first hour after dosing and R_{in} is stimulated later on causing an increase in neutrophil count after chronic dosing. In addition, the elimination clearance is inhibited by the effect.

5. PROTEIN BINDING OF PROTEIN THERAPEUTICS

The binding of drugs to circulating plasma proteins can influence both the distribution and clearance of drugs, and consequently their pharmacodynamics. Since it is generally accepted for small drug molecules including small proteins that only the unbound drug molecules can pass through membranes, distribution and elimination clearances of total drug are usually smaller than those of free drug. Accordingly, the activity of the drug is more closely related to the unbound drug concentration than to the total plasma

concentration. For other protein drugs however, plasma binding proteins may act as facilitators of cellular uptake processes, especially for drugs that pass membranes by active processes. When a binding protein facilitates the interaction of the protein therapeutic with receptors or other cellular sites of action, the amount of bound drug influences the pharmacodynamics directly.

Numerous examples of binding proteins are reported for proteins: IGF-I and IGF-II, t-PA, growth hormone, DNase (63), nerve growth factor, etc. (64). Some proteins have their own naturally occurring binding proteins that bind the protein specifically. As an example, six specific binding proteins are identified for IGF-I, denoted as IGFBP-1 to IGFBP-6 (65,66). The IGFBPs are high affinity, soluble carrier proteins that transport IGF-I (and IGF-II) in the circulation (66). In humans, IGFBP-3 appears to be the most important binding protein for IGF-I since it is the most abundant in serum and tissues. At least 95% of the total human serum concentration of IGF-I is bound to IGFBP-3 (67). IGFBP-3 seems to act as a reservoir for IGF-I, and as such to protect the organism against acute insulin-like hypoglycemic effects. Indeed, the hypoglycemic effect is related to the free IGF-I plasma concentration. In this case, the binding protein limits the accessibility of IGF-I to receptors since all binding proteins have substantially higher affinities for IGF-I than the IGF receptors (68). In contrast, the delayed, indirect effects of IGF-I, such as its anabolic effects, may be related to the bound IGF-I levels. This is supported by evidence that the IGFBPs may play an active role in the interaction with target cells, and may act as facilitators for the delivery of IGF-I to certain receptors (66). One example is the demonstration that the affinity of the binding protein for IGF-I (IGFBP-6) at the cell surface is lower than in solution, which would make it easier for IGF-I to leave its association with the binding protein and to engage in binding with a cell-based receptor. As such, the IGFBPs may act as inhibitors for certain IGF-I effects, and as stimulators for other IGF-I effects.

It is demonstrated that the elimination half-life of bound IGF-I is significantly prolonged relative to that of free IGF-I (64,69,70). This suggests that unbound IGF-I only is available for elimination by routes such as glomerular filtration and peritubular extraction. The binding proteins for IGF-I are also responsible for the complicated pharmacokinetic behavior of IGF-I. The IGFBPs can be saturated at high IGF-I plasma concentrations, typically reached after endogenous therapeutic administration of IGF-I. At high doses, the binding proteins saturate and leave a larger proportion of free protein available for elimination. Additionally, the nonlinear pharmacokinetics of IGF-I are complicated by the fact that the concentrations and relative ratios of the IGFBPs change with time during chronic dosing. The binding proteins are also very different between species, which makes interspecies scaling of the IGF-I pharmacokinetics for IGF-I impossible.

Another example is growth hormone (GH), for which a specific high-affinity binding protein homologous with the extra cellular domain of the growth hormone receptor is present in human plasma (71,72). At least two GH-binding proteins (GHBP) have been identified in plasma with respectively high and low binding affinities for GH (64). Growth hormone binding protein binds about 40–50% of circulating GH at low GH concentrations of about 5 ng/mL (73). At higher circulating GH levels, the binding proteins become saturated (Fig. 11). The clearance of bound GH is about 10-fold slower than that of free GH (74). Consequently, the binding proteins prolong the elimination half-life of GH, and as a result, enhance or prolong its activity. On the other hand, plasma binding of GH prevents access of free GH to its receptors, and this could decrease its activity (64).

Other protein therapeutics seem to bind to circulating proteins in a more nonspecific way. As an example, a recombinant derivative of hG-CSF, nartograstim, showed 92% binding in rat plasma, presumably to albumin (35).

6. INTERSPECIES SCALING

Techniques for the prediction of pharmacokinetic parameters in one species from data derived from other species have been applied for many years (75,76). Such scaling techniques use various allometric equations based on body weight (see Chapter 2). The following allometric equation is routinely employed:

$$P = a.W^b$$

where P is the pharmacokinetic parameter being scaled, W is the body weight, a is the allometric coefficient, and b is the allometric exponent. Although a and b are specific constants for any compound and for each pharmacokinetic parameter, the exponent b seems to average around 1 for volume terms such as the volume of distribution and 0.75 for rates such as elimination and distribution clearances. Since the elimination half-life of any drug is proportional to the volume of distribution and inversely proportional to the elimination clearance, b is about 0.25 for elimination half-lives. Allometric scaling of pharmacokinetic parameters has been difficult for small synthetic drug molecules, especially for those drugs with a high hepatic clearance and quantitative and/or qualitative interspecies differences in metabolism. In contrast, the biochemical and physiological processes that are responsible for the pharmacokinetic fate of biologics such as peptides and proteins are better conserved across mammalian species. As such, allometric scaling for those compounds has been more reliable and accurate (77). It is our experience that the systemic exposure in humans of proteins that follow linear pharmacokinetics can be predicted within a factor of two from pharmacokinetic data from 3 to 4 animal species. As a typical example, we could

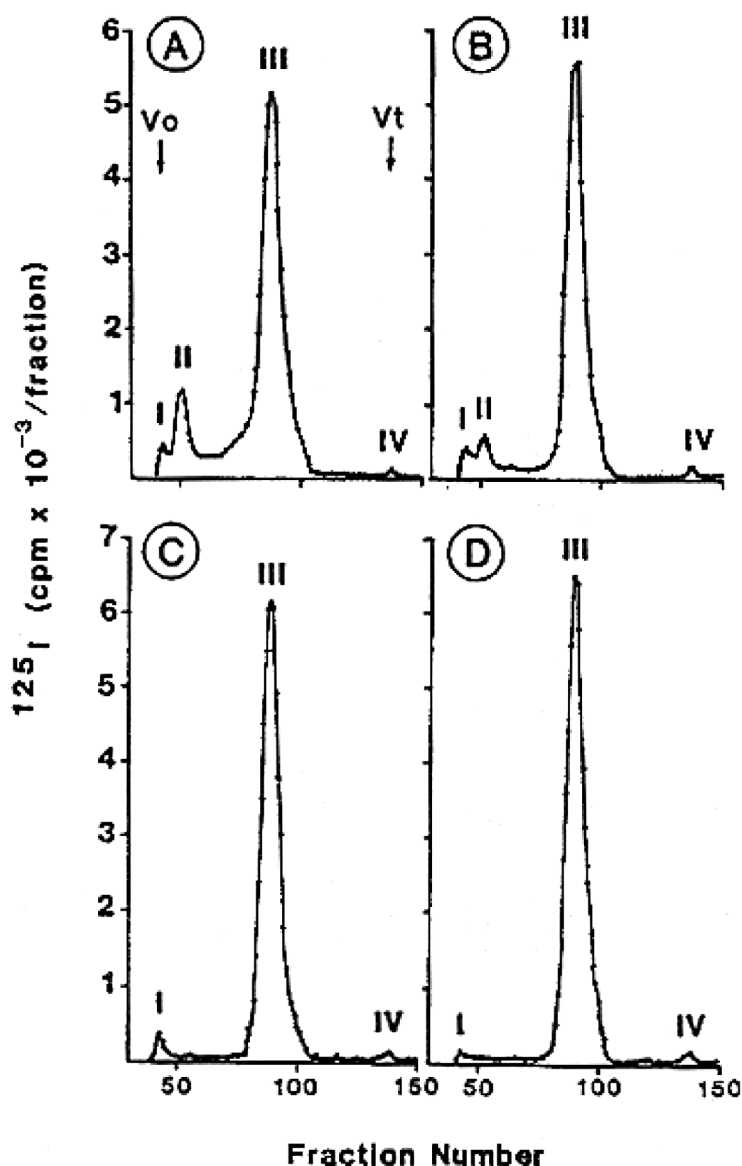


Figure 11 Gel filtration profiles of ^{125}I -hGH in plasma on Sephadex G-100. V_0 and V_t are the void and total volumes, respectively. A. Blank plasma with endogenous level of hGH only; B. One hundred and twenty six nanogram per milliliter hGH added; C. Ten nanogram per milliliter hGH added; D. Tracer only (no plasma). Peak III corresponds to monomeric hGH; peak II and the plateau region between peaks II and III refer to the plasma-bound hGH; peak IV is free iodide. Higher hGH concentrations saturate the binding proteins as peak II becomes smaller relative to peak III (C vs. B vs. A). (From Ref. 66.)

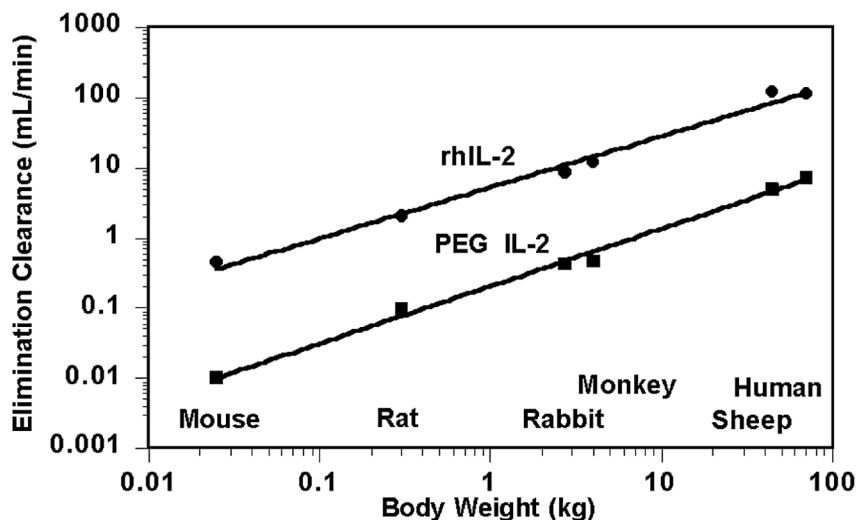


Figure 12 Allometric interspecies scaling of the elimination clearance of IL-2 and PEG IL-2.

scale the pharmacokinetic parameters for IL-2 and PEG IL-2, as demonstrated in Fig. 12, for the elimination clearance. Notice that the regression lines for both compounds are parallel, which is expected if PEGylation decreases the clearance to the same degree in all species.

A helpful although potentially less accurate prediction can be made based on pharmacokinetic data from one species to another based on the average allometric exponents for volumes and clearances. Interspecies scaling is helpful in the prediction of doses for pharmacological animal models of disease, toxicology studies, and the first human studies. Indeed, if the efficacious concentration of a protein drug is known from *in vitro* studies, one might predict the dose needed to reach these levels in an animal efficacy or toxicology model when pharmacokinetic data are known from another species. Similarly, if an estimation of the maximum tolerated exposure can be made, allometric scaling may be helpful to determine the highest dose in toxicology studies. The dose that results in efficacious concentrations may be taken as the lowest dose in toxicology studies. Additionally, the efficacious dose in humans can be estimated from the animal pharmacokinetic data. A starting dose in the first human study (usually a dose-escalation study) can be chosen as this estimated efficacious dose, divided by a factor of two or more, based on conservative safety considerations.

It needs to be emphasized that allometric scaling techniques are useful tools to predict a dose that will assist in the planning of dose-ranging studies, but are not a replacement for such studies. The advantage of

including such dose prediction in the protocol design of dose-ranging studies is that a smaller number of doses need to be tested before finding the final dose level. Interspecies dose predictions simply narrow the range of doses in the initial pharmacological efficacy studies, the animal toxicology studies, and the human safety and efficacy studies.

7. HETEROGENEITY OF PROTEIN THERAPEUTICS

The identity, purity, and potency of small synthetic drugs can be demonstrated analytically, and consequently, they are usually completely defined in terms of their chemical structure. Peptides, proteins, and other biotechnologically derived compounds are usually more complex compounds, and it is generally not possible to define them as discrete chemical entities with unique compositions. The physicochemical and biochemical characteristics of proteins are not only dependent on the amino acid sequence (primary structure), but also on the shape and folding (secondary and tertiary structures), and the relationship between the protein molecules themselves, such as the formation as aggregates (quaternary structure). Biotechnologically-derived and endogenous proteins may be heterogeneous at each structural level. For natural IFN-gamma, for example, six naturally occurring C-terminal sequences have been identified (78–80).

In addition post-translational modifications of proteins, such as the degree of glycosylation of amino acid residues, may be different. The secreted and membrane-associated proteins of almost all eukariotic cells are glycosylated (81,82), and different glycoproteins have also different carbohydrate contents, from ~3% for serum IgG to >40% for erythropoietin (EPO). Erythropoietin has three *N*-linked and one *O*-linked sugar chains. The degree of glycosylation differs according to the cell line used for production. For example, GM-CSF and M-CSF are nonglycosylated in bacterial cell lines such as *E. coli*, moderately glycosylated in yeast, and heavily glycosylated in mammalian cell lines. Receptor binding studies with GM-CSF have shown that the receptor affinity decreases with an increase of the level of glycosylation (83).

Another classical example is recombinant human tissue-plasminogen activator (t-PA). Although the active enzyme was first derived from *E. coli* cultures, this cell line lacks several desirable biological activities, such as glycosylation ability and the ability to form the correct three-dimensional t-PA structure. Finally, recombinant t-PA was cloned into a Chinese hamster ovary (CHO) cell line. These mammalian cells carried out the glycosylation, disulfide bond formation, and proper folding similar to human cells (84).

Besides the importance of correct glycosylation for activity, differences in glycosylation may also have an influence on the pharmacokinetics. A typical example is that the removal of terminal sialic acid residues from

the sugar chains of EPO (asialo-EPO) causes complete loss of *in vivo* biological activity, but increases *in vitro* activity. The loss of *in vivo* activity of asialo-EPO was explained by a rapid removal from the systemic circulation, which resulted from hepatic uptake mediated by galactose-recognizing receptors.

8. CHEMICAL MODIFICATIONS OF PROTEIN THERAPEUTICS

Besides the mostly unwanted heterogeneity of protein drugs introduced by the manufacturing process, other chemical modifications of protein and peptide drugs are intentional to obtain molecules with specified characteristics. Variant proteins can be engineered that differ from natural proteins by exchange, deletion, or insertion of single amino acids, or longer sequences up to entire domains. Small changes in the chemical structure of proteins may cause differences in pharmacokinetics and pharmacodynamics. In addition, mutations may affect glycosylation patterns and conformational changes, which in turn may affect clearance and receptor interactions. A single amino acid mutation in t-PA or the removal of carbohydrate on a single amino acid in t-PA resulted in plasma concentration profiles that were very different from natural t-PA (Fig. 13) (85).

Modification of peptide and protein drugs with the aim of changing the pharmacological activity may at the same time affect the pharmacokinetic behavior of the molecules. In other instances, the increase of duration of response may be exclusively attributed to a change in the pharmacokinetics such as an increase in residence time. Such modifications include amino acid substitution, deletions and additions, cyclization, drug conjugation, glycosylation or deglycosylation, etc.

The elimination half-life of many peptide and protein drugs is rather small. Consequently, frequent dosing or continuous infusion is necessary to maintain efficacious plasma levels of the drug. Several approaches have been applied to decrease the elimination clearance of biotechnological drugs. One approach is chemical modification such as PEGylation, i.e., the attachment of monomethoxy polyethylene glycol polymer (PEG) to the protein. An example is PEG IL-2, which usually consists of a mixture of rhIL-2 molecules (Mw 15 kDa) with 1–5 or more PEG polymers attached to each molecule on the α -amino portions of the lysine residues. The production process determines the average number of PEG residues attached, but any process results in a mixture. With each PEG addition, the molecular weight increases with about 7 kDa, but because of the attraction of water molecules, the hydrodynamic size increases even more (95–250 kDa). Increasing the degree of PEGylation decreases the elimination clearance and the volume of distribution (Fig. 14). Since the elimination clearance usually decreases relatively more than the decrease in volume of distribution, the elimination half-life of PEG IL-2 is longer than for IL-2. Based

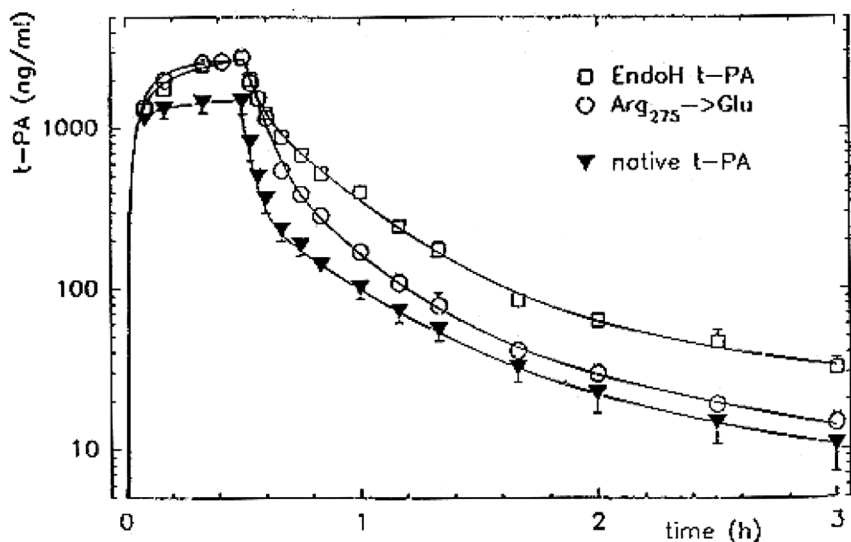


Figure 13 t-PA plasma concentrations after 30-min IV infusions of 0.6 mg/kg t-PA in groups of 4 rabbits. The figure shows the marked effect on clearance of a single amino acid mutation (Arg₂₇₅->Glu) or of removal of high mannose carbohydrate at Asn₁₁₄ by the enzyme endoglycosidase H (EndoH t-PA), as compared to native t-PA. (From Ref. 85.)

on the relationship between elimination clearance and effective molecular weight, it is possible to calculate the optimal degree of PEGylation to obtain the desired systemic exposure (86,87).

The effect of prosthetic sugar groups on elimination and targeting is illustrated by the comparison of the pharmacokinetics of native glucose-oxidase (GO), deglycosylated GO (dGO), and galactosylated GO (gGO) in mice (88). A saturable mechanism was responsible for GO and dGO uptake by mononuclear phagocytes, although there was a substantial difference in elimination half-life (10 min for GO; 100 min for dGO). In contrast, gGO had a half-life of 4 min and was taken up preferably by hepatocytes, presumably through hepatic galactose receptors. This is an example where receptor-mediated endocytosis through sugar-recognizing receptors is an efficient hepatic uptake mechanism for glycoproteins. However, when terminal sialic acid residues on the carbohydrate moieties of glycoproteins shield the receptor-binding sugars, hepatic receptor-mediated endocytosis is lower than the desialylated analogues (21). This has been demonstrated for rEPO and rGM-CSF (29). The protection by sialic residues appears to be a natural mechanism essential for the normal survival of enzymes, acute-phase proteins (such as α_1 -acid glycoprotein), and most plasma proteins of the immune system.

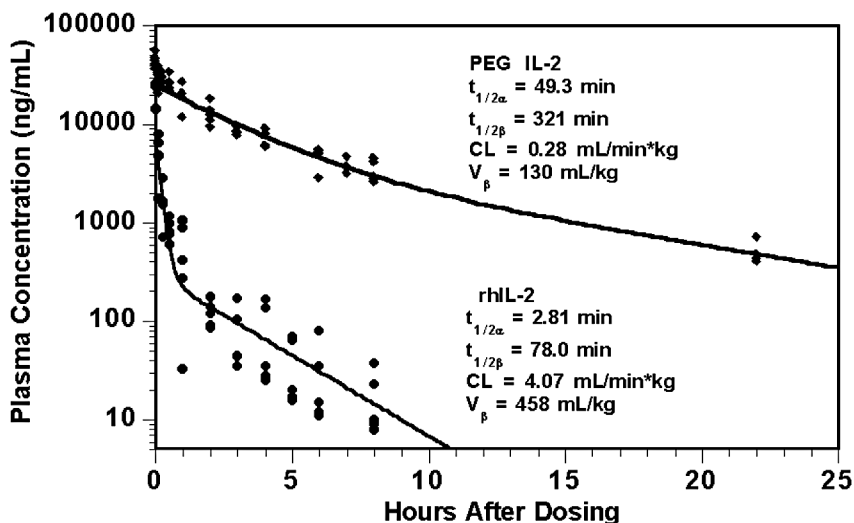


Figure 14 Pharmacokinetics of recombinant human interleukin-2 (rhIL-2) and its PEGylation form (PEG IL-2) in rats after IV bolus administration of 0.25 mg/kg. The data were described by a linear two-compartmental pharmacokinetic model.

9. IMMUNOGENICITY

Immunogenicity is the ability to induce the formation of antibodies, a prerequisite for antigenicity, which is the ability to react with specific antibodies. Immunogenicity is an important property distinguishing most biologic products from most small drug molecules. An immunogenic response to heterologous (nonhost) proteins is expected, as antibody formation is also often observed after chronic dosing of human proteins in animal studies. However, recombinant human proteins may also stimulate the production of circulating antibodies in chronic human therapy and clinical studies. In this case, immunogenic responses are sometimes associated with the formation of protein aggregates, altered proteins forms or fragments, such as acetylated protein or proteins with broken disulfide bridges (for interferons, for example). In other cases, impurities from cell substrates or media components are either directly immunogenic or act as adjuvants to stimulate antibody formation against the protein.

Immunogenic responses can cause a wide variety of unwanted effects, with different degrees of severity. Safety issues include the potential for injection site reactions, systemic hypersensitivity reactions, and anaphylactic shock in some cases. As an example, bovine Cu, Zn-superoxide dismutase (Cu, Zn-SOD) (Orgotein) as a treatment for various arthritic diseases was withdrawn from several European countries because of hypersensitivity.

Asparaginase from bacterial origin (*E. coli*), indicated in the therapy of acute lymphocytic leukemia, causes a very high level of allergic reactions (3–73% incidence) (89). The manufacturer of asparaginase has a scheme for skin testing and desensitization should skin tests be positive prior to therapy. Another one of the few nonhuman proteins on the market is the thrombolytic streptokinase, produced in group C β -hemolytic streptococci. Levels of antistreptokinase antibodies can be present in patients as a result of a recent streptococcus infection, and therefore, allergic reactions have been noticed (1–4% incidence), some anaphylactic and anaphylactoid responses (89). The manufacturer cautions against readministration within a period of 5 days to 12 months of either administration of streptokinase or the development of a streptococcus infection. Human antibodies have been observed to recombinant human proteins for human interferons (IFN), human growth hormone (hGH), human insulin, and human factor VIII. Hypersensitivity reactions are however rather rare. In general, for human recombinant proteins, immunogenicity has not been the primary limitation for their clinical use; poor PK and PD are frequently the major obstacles for efficacy.

Immunogenicity can be a problem in the study (and use) of protein drugs since the presence of antibodies can complicate the interpretation of preclinical and clinical studies by inactivating (neutralizing) the biological activity of the protein drug. Additionally, protein–antibody complex formation may affect the distribution, metabolism, and elimination of the protein drug. Neutralizing antibodies may inactivate the biological activity of the protein by blocking its active site or by a change of the tertiary structure by steric effects. Antibodies are most likely to be induced when the protein is foreign to the host. Examples of such situations are when mouse-derived monoclonal antibodies are administered to humans, or when human recombinant proteins are tested for safety in animals. Extravascular injections (e.g., s.c. and i.m.) are also more likely to stimulate antibody production than intravenous administrations, presumably because of the higher degree of protein precipitation and aggregation at the injection site. This was demonstrated for IL-2 (90) and INF- α (91,92).

Antibodies may directly neutralize the activity of the protein. This has been observed for interferons in the presence of neutralizing IgG, for example. If neutralization occurs, it indicates that at least some fraction of the antibody population binds at or near the active site, which blocks activity (93). Irrespective of the neutralizing capabilities of the antibodies formed, they may also indirectly affect the efficacy of a protein drug by changing its pharmacokinetic profile (Fig. 15). Elimination clearances of protein drugs may be either increased or decreased by antibody formation and binding. An increase of the clearance is observed if the protein–antibody complex is eliminated more rapidly than the unbound protein (94). This may occur when high levels of the protein–antibody complex stimulate its

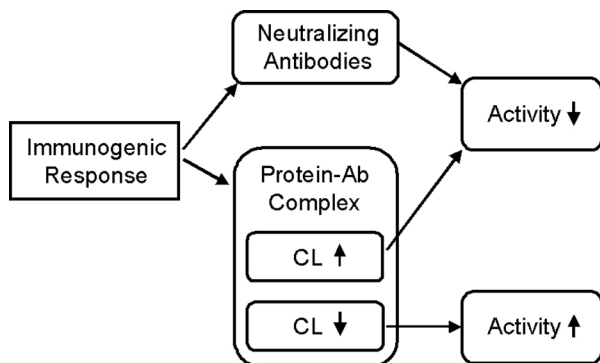


Figure 15 Effect of antibody formation on pharmacokinetics and pharmacodynamics of protein drugs.

clearance by the reticuloendothelial system (95). In other situations, the serum concentration of a protein can be increased if binding to an antibody slows down its rate of clearance, because the protein–antibody complex is eliminated slower than the unbound protein (93). In this case, the complex may act as a depot for the protein and, if the antibody is not neutralizing, a longer duration of the pharmacological action may occur. For example, the clearance of rIFN- α 2a in cancer patients was increased because of an antibody response. In contrast, human leukocyte INF- α in rats was decreased 15-fold when circulating antibodies were present. A decrease of clearance in the presence of antibody titers was also detected for t-PA in dogs (93).

Both an increased and decreased clearance is possible for the same protein, dependent on the dose level administered. At low doses, protein–antibody complexes delay clearance because their elimination is slower than the unbound protein. In contrast, at high doses, higher levels of protein–antibody complex result in the formation of aggregates, which are cleared more rapidly than the unbound protein.

The most worrisome situation occurs when neutralizing antibodies are formed during chronic therapy with a protein drug, and when the antibodies cross-react with the endogenous protein or another endogenous factor (89). This is especially a safety concern if the endogenous protein has a unique type of activity, and there is no redundant mechanism to compensate for the activity loss of the neutralized factor. As an example, humans dosed with thrombopoietin (TPO) developed long-term thrombocytopenia, which is believed to be caused by the neutralizing activity of antibodies against endogenous TPO (89). Apparently, EPO is the only factor really important for the formation of platelets. Some patients appeared to have pre-existing

antibodies to TPO. Pre-existing antibodies were also detected for interferons in cancer and HIV patients.

Besides route of administration and product characteristics, other immunogenic determinants are dose and regimen, disease, and concomitant medications. Typically, larger proteins are more immunogenic than smaller ones. The effect of dose size on the antibody response is unpredictable, although cumulative dose may be more important than the daily dose. With interferons, for example, a higher cumulative dose resulted in less neutralizing antibodies. Time, more so than dosing frequency, is important, since any antibody response needs weeks to months to develop fully. In humans, IgM levels appear after 5–7 days, while IgG serum concentrations peak 3–4 weeks after dosing initiation. Patients with infectious diseases, presumably because of a stimulated immune system, showed higher antibody levels than cancer patients, who are typically immunosuppressed. Similarly, autoimmune disease state is a factor that might stimulate immunogenicity responses, while a lower response is possible in patients with kidney and liver disease. Immunosuppressants such as cyclosporin as concomitant medication may diminish the immunogenic response.

Because of the different possible effects of an immunogenicity response on the PK/PD of protein drugs, the study of an antibody response is very important in the drug development process. However, the presence of an immunogenic response in animal studies is rarely a prediction of a similar occurrence in humans. More importantly, the value of certain preclinical toxicology studies may be questioned when large titers of neutralizing antibodies are measured, because a lack of toxicity findings may be caused by the neutralization of the toxicodynamic effect. For the situation in humans, the measurement of antibody, and neutralizing antibody titers, in chronic clinical studies is important.

10. CONCLUSIONS AND IMPLICATIONS FOR PRECLINICAL DRUG DEVELOPMENT

In summary, the PK/PD of biotechnologically derived molecules is unique and amenable to mechanistic evaluations. These evaluations provide sound fundamental background for extrapolation across species and for prediction of outcomes under various dosing regimens.

Proteins, and chemically modified proteins—including glycoproteins—often possess similar absorption, distribution, and elimination mechanisms across species. Through understanding differences in physiology and anatomy of those species, systems analyses can be conducted to extrapolate findings into predicted human outcomes.

Similarly, when the PK/PD of these molecules have been characterized in humans, with the support of the preclinical database, one can predict outcomes when doses, routes of administration, and dose frequencies are

modified. It becomes particularly important in human evaluations to understand the mechanism of elimination since it is common for manufacturing changes to occur in the clinical or commercial setting. Here, the preclinical database provides invaluable insight into potential changes in human efficacy or safety.

Antigenicity remains a unique and often troublesome property of these molecules. While antigenicity can result in simple binding complexes, they can also neutralize the pharmacologic activity of the molecule and may cross-react with endogenous or similar molecules. These latter responses can result in profound and chronic toxicity. Understanding the outcome of induced antibodies on the PK/PD of large molecules in preclinical models provides an understanding of safety that cannot be studied in humans.

While the issues of large molecule drug development are unique from small molecules, those issues can be challenging and complex. Nevertheless, biotechnology has proven itself as a realm of therapeutic intervention that can treat some of our most daunting and destructive diseases. Indeed, our understanding of these diseases, the mechanisms by which we can modulate disease pathways and the technology around development science will continue to fuel the success of biotechnology.

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Preclinical Pharmacokinetic– Pharmacodynamic Modeling and Simulation in Drug Development

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1. INTRODUCTION

The pharmacokinetic literature is replete with examples of pharmacokinetic–pharmacodynamic models developed using animals. When those models can be extrapolated to the human condition, their value becomes significant. Otherwise, those models may still add to the (patho)physiology body of knowledge, but as far as the drug development process is concerned, they are simply exercises in modeling. Many books have been written on pharmacokinetic modeling, the most cited reference being Gibaldi and Perrier (1), and many excellent reviews have been written on pharmacodynamic models and modeling, including Derendorf and Meibohm (2) and Sheiner and Steimer (3). It would be redundant and add little to the scientific literature to re-present that material in a new format. Instead, the purpose of this chapter will be to review some interesting published case studies where the pharmacokinetic–pharmacodynamic models developed preclinically helped guide clinical drug development.

2. WHAT IS A MODEL AND WHY DO WE DO WE MAKE THEM?

A system is a collection of interacting objects. A car, a computer, and a living organism each represent different types of systems. A model is any representation of a system that accounts for the properties of the system. Certainly, many classes of models exist. One that comes immediately to mind is a scale model wherein some physical object is re-created and scaled to a size that is more convenient for viewing, e.g., an architect's design of new building. In pharmacokinetic–pharmacodynamic modeling, the models are mathematical and statistical in nature, and they attempt to characterize the relationship between dose and some dependent variable. A pharmacokinetic model describes the relationship between dose and drug concentration, usually in plasma or serum, while the pharmacodynamic model relates drug concentration to efficacy, adverse events, or outcome. A pharmacokinetic–pharmacodynamic model integrates dose to response and the role of such models in drug development is the subject of a guidance issued by the Food and Drug Administration (4).

Modeling, especially quantitative modeling based on mathematics and statistics, serves many useful purposes. One is that it characterizes and summarizes a set of data into a cohesive structure. For example, given a set of concentration–time data, a pharmacokinetic model summarizes the data into a few simple parameters; for example, clearance—a parameter that relates concentration to rate of change in concentration. Second, and most importantly, is that modeling may allow predictions to be made, a process that is referred to as simulation. Conditional on a pharmacokinetic model structure and parameter values or population distributions, predictions can be made regarding changes in dose frequency or total dose administered. Given a pharmacokinetic–pharmacodynamic model, predictions on outcome or safety can be made regarding projected changes in dose, dose frequency, or changes in the parameters that describe the system, such as the expected increase in exposure if renal clearance were decreased in patients with renal failure. The ability to predict makes simulation a very important tool in drug development, since further product development can then proceed in a rational manner; the converse of this would be to have to do study after study to answer important questions that arise during the drug development process, without being able to integrate answers to the questions in a coherent, testable whole.

3. WHAT CONDITIONS ARE NECESSARY FOR THE PRECLINICAL MODEL TO BE VALID IN HUMANS?

Most of the literature that advocates an increased role of pharmacokinetic–pharmacodynamic modeling in the drug development process has focused on developing models early in *clinical* development and then using these

models to help develop and choose doses for later phases in development. Using *preclinical* models to help guide clinical development has been less advocated because there is always a leap of faith made when extrapolating information obtained in animals to humans.

Not all preclinical models can be successfully extrapolated to the humans and there are a number of conditions that should be met before placing any credibility on the extrapolation. First, drug concentrations should be measured using a validated pharmacokinetic assay. Does the assay need to be validated according to Good Laboratory Practices (GLPs) or the Guidance to Industry issued by the Food and Drug Administration on Bioanalytical Method Validation? No, unless the data will be used to support a New Drug Application. If the bioanalytical method is not GLP per se, it should be sufficiently precise and accurate for the results to have scientific value. Second, ideally the pharmacodynamic assay needs to be validated as well. Validation of pharmacodynamic assays is straightforward if the biological entity (biomarker) being measured is easily quantified, such as with laboratory parameters like plasma glucose or hemoglobin A1c, or an ECG parameter like QTc intervals. However, validation of assays that generate “soft” data, like any behavioral pharmacology measurement, is much more difficult. Also, with a truly novel drug target, validation of the biomarker may not be possible or the link between the marker and the disease process may not be firmly established, i.e., the marker may be speculative (5,6). Like a bioanalytical assay for drug concentrations, the pharmacodynamic assay results must be accurate, precise, and repeatable. A complementary aspect of pharmacodynamic assay validation that is even more important than the assay per se is to be able to unequivocally link the measured biological entity to a biomarker with a very specific role on the disease progression and the therapy under investigation. It is not uncommon to be able to precisely measure putative pharmacodynamic parameters that are later found to be not related to the disease. Normally, the link between pharmacodynamic assay and biomarker is done through the establishment or collection of relevant scientific evidence, but it should be done early in the development process.

Ideally, a robust link between pharmacokinetics and pharmacodynamics needs to be established. Greater confidence is placed in models where the relationship between drug concentrations and pharmacodynamic effect is straightforwardly established and can be seen by simple inspection, such as when established paradigms like a linear model or $E_{\max}$ model are appropriate. Analysts and project team members may then more readily accept the outcome. Some drugs have no apparent concentration–effect relationship, such as the relationship between drug concentration and effect for antidepressants or antipsychotics, so pharmacokinetic–pharmacodynamic modeling may not be even be possible for these drugs. Model credibility is inversely proportional to the degree of mathematical and statistical manipulation that goes into establishing the relationship between drug

concentration and pharmacodynamics. Also, the greater the number of assumptions that go into a model, the more likely it is that some of these assumptions are wrong or hard to convincingly support. It is hard to overstate the importance of justifying any and all assumptions embedded in a pharmacokinetic–pharmacodynamic model. The impact of possible inaccuracies must be persuasively assessed before a model will be accepted as credible.

Even if a well-defined relationship between drug concentration and effect is established in animals, there is no guarantee the relationship will hold in humans. We all understand that nonhuman animal species and humans are different at many levels, so making the extrapolation from animals to man becomes a leap of faith implying that animals and man are more similar than dissimilar. The more dissimilar the pharmacology/physiology between the animal species and humans, the more tenuous the extrapolation. Hence, the physiology of the system under study should be understood and species differences must be identified and corrected for during the extrapolation process. Information already independently established and part of the scientific body of peer-reviewed literature can be proactively used to support it, for example through targeted collaboration with academic institutions. From within a drug development company, lead compounds that have previously had a pharmacokinetic–pharmacodynamic model established preclinically, and then tested and validated in humans provide a great help in this regard. This provides some experience in the validity of the model and extrapolation, should the lead compound fail and back-up compounds having the same mechanism of action have to be created or resurrected from pipeline purgatory.

4. CASE STUDIES

The remainder of this chapter will deal with case studies where pharmacokinetic–pharmacodynamic models established preclinically were used to help guide clinical development or answer key development questions that could not be addressed in humans.

4.1. Case Study 1: *Modeling the EEG Effect of Drugs Affecting the Central Nervous System*

Meindert Danhof at the Center for Bio-Pharmaceutical Sciences at the University of Leiden in The Netherlands has been one of the leading proponents of preclinical pharmacokinetic–pharmacodynamic modeling as a means to accelerate drug development through the use of rational dose selection, so it seems natural to review some of his research first. Most of this body of research has revolved around the use of the electroencephalograph (EEG) as a tool to provide relevant biomarkers for agents affecting the central

nervous system (CNS) in rats, benzodiazepines in particular, and then comparing these results to the results in humans. In many instances, the concentration that produces 50% of the maximal effect (EC_{50}) in rodents is similar to the EC_{50} in man, thus supporting the statement that the drug has similar potency in both species. Once an EC_{50} has been established for a drug, this concentration becomes the target to achieve in humans.

As an example of the methodology Della Paschoa et al. (7) used pharmacokinetic–pharmacodynamic modeling to characterize the anticonvulsant and EEG effects of phenytoin in rats. Male, Wistar rats were separated into two groups. Group I was implanted with EEG electrodes one week before dosing, whereas Group II was implanted with electrodes to induce seizures. Phenytoin 40 mg/kg was intravenously infused for 5 min with blood samples collected for 6 hr for drug concentration analysis. EEG recordings were continuously measured in Group I for 5 hr, and the drug-induced change in the total number of waves in the 12.0 to 30.0 Hz frequency was used as a biomarker. Seizures were induced in Group II by increasing the amplitude of an electrical pulse to the frontal cortex until seizures occurred. Seizures were induced five times prior to dosing to establish a baseline and periodically thereafter for up to 2 h after dosing. The elevation of seizure threshold above baseline was chosen as a biomarker for anticonvulsant effect.

To tackle model-based analysis of this substantial body of data, the pharmacokinetics of phenytoin were characterized first. Phenytoin concentrations were described using a two-compartment model with Michaelis–Menten elimination kinetics. Second, phenytoin pharmacokinetics were linked to the pharmacodynamics. The observed hysteresis between EEG effect and phenytoin concentration was collapsed through the use of an effect compartment where the parameters related to transfer to the effect compartment were modeled using a nonparametric convolution procedure (Fig. 1). EEG effects (E) were then modeled using a sigmoid $E_{\max}$ model.

$$E = E_0 + \frac{E_{\max} \cdot C_e^n}{EC_{50}^n + C_e^n} \quad (1)$$

where E_0 is baseline effect, $E_{\max}$ is maximal effect, C_e is the effect site concentration, EC_{50} is the concentration that produces 50% the maximal effect, and n is the Hill parameter. Anticonvulsant effects were also modeled using a sigmoid $E_{\max}$ model under the assumption that $C_e \ll EC_{50}$. After simplification of Eq. (1) the resulting model was

$$E = E_0 + B^n \times C_e^n \quad (2)$$

where B is the ratio of $E_{\max}$ to EC_{50}^n . The observed and model predicted fits to the EEG and anticonvulsant data are shown in Fig. 2. The EC_{50} for EEG effect was estimated at $12.6 \pm 1.3 \mu\text{g/mL}$ (mean $\pm$ standard error). Correcting for protein binding in the rat ($\sim 30\%$ unbound), the EEG effect occurred

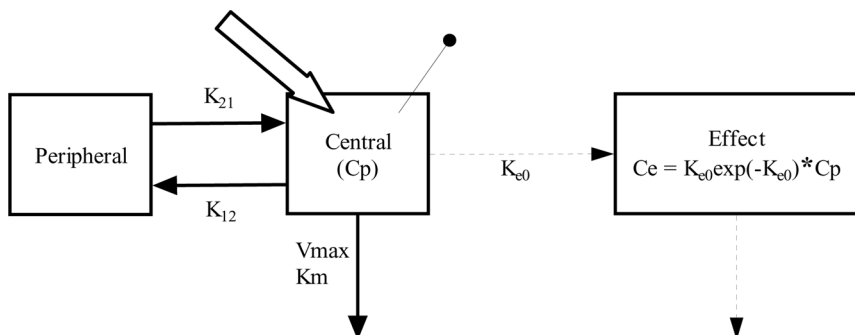


Figure 1 Model describing the pharmacokinetics and pharmacodynamics of phenytoin. Legend: K_{21} and K_{12} are the rate of transfer from compartment 2 to compartment 1 and from compartment 1 to compartment 2, respectively, $V_{\max}$ is the maximal rate of elimination, K_m is Michaelis constant, K_{e0} is the effect compartment transfer coefficient, C_p is the observed plasma concentration, and C_e is the hypothetical effect compartment concentration. Solid lines with arrows denote mass transfer. Dashed lines with arrows denote hypothetical transfer. The '*' operator denotes the convolution integral. The large arrow denotes the compartment where dosing occurs. The solid lines with solid circle denote the sampling compartment. (Modified from Ref. 7. Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. Copyright, 1998.)

in the range of 1.8–6.0 $\mu\text{g/mL}$, which is the same concentration range needed for therapeutic effect in humans. The elevation of the threshold for seizure activity also occurred in a similar range, 1.75–10 $\mu\text{g/mL}$. This finding, similarity in potency between rats and humans, has been seen with many other drugs including the opioids alfentanil, fentanyl, and sufentanil (8).

This methodology can also be used to answer other development questions. For instance, Cox et al. (9) reported that GR90291, the major metabolite of remifentanyl, an ultra-short acting opioid analgesic, had such low potency and brain penetrability that its expected contribution to the total anesthetic effect after remifentanyl administration in humans was of little significance. Parker et al. (10) used this methodology to assess the pharmacodynamic profile of S 16924 and S 18327, two potential antipsychotic agents, and found that both test compounds had a unique pharmacologic profile compared to clozapine. Tuk et al. (11) demonstrated that α -hydroxy-midazolam, the major metabolite of midazolam, competed with its parent for the same receptor site. The metabolite had the same maximal effect as the parent, but was 7 to 8-times less potent, indicating that both compounds act as full agonists at benzodiazepine binding sites.

The pharmacokinetic–pharmacodynamic approach in this case is clearly promising. The advantages to the methodology include, but are not limited to (1) its use when studies cannot be done in humans, such as

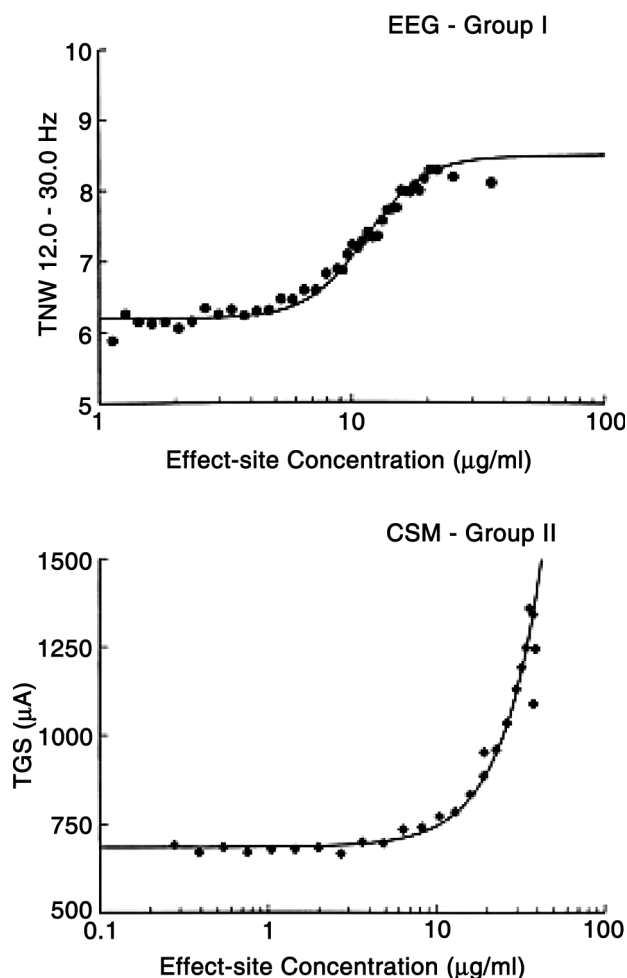


Figure 2 Pharmacokinetic–pharmacodynamic relationship between phenytoin concentration at the effect site and EEG effect (top) and anticonvulsant effect (bottom). The solid lines represent the predicted effect based on the respective pharmacokinetic–pharmacodynamic model. Legend: CSM, cortical stimulation model. (Data presented by Della Paschoa et al. (7). Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. Copyright, 1998.)

the study involving the metabolite of remifentanyl, (2) its potential as a screen in drug discovery by aiding in the choice of drugs with appropriate potency, (3) its use as screen for drug interactions, and (4) its substantial capability to explain observations from a variety of sources. For the particular case, we have selected here, the disadvantage is that EEG measurements require special surgical techniques and access to a rat EEG machine,

data analysis requires experienced modelers, and the entire approach, if not kept in check, may quickly become costly and time-consuming, and thus of limited utility.

4.2. Case Study 2: Dose Selection for Phase 1 and 2 Clinical Trials

Gomeni et al. (12,13) reported on the use of modeling and simulation to help select the doses for a first-time-in-man (FTIM) and proof of concept study for a new unspecified agent that affects the CNS. Pharmacokinetic and plasma protein binding information was available in rats, cynomolgus monkeys, and dogs as part of the toxicology program. Protein binding was also estimated in human plasma. Rodents and rhesus monkeys were studied in pharmacology efficacy studies. Only the pharmacodynamic response was available in rhesus monkeys, whereas pharmacokinetics and pharmacodynamics were available in the rodent pharmacology study. In vitro receptor binding studies were done in rhesus monkeys and man to compare the binding affinity relative to rodents.

Certain assumptions were made during the course of the analysis. First, unbound concentrations were hypothesized to be a better predictor of response than total concentrations. This assumption is a common one since only unbound (free) drug tends to cross the blood–brain barrier, unless the drug shows receptor-mediated transport into the brain, which is not that common for small molecules. Second, it was assumed that receptor binding in the brain was directly proportional to the pharmacodynamic effect measured in behavioral tests administered to rats. Hence, measurement of receptor binding could be used as a biomarker for pharmacodynamic activity. Third, the pharmacokinetics of the system were assumed linear and independent of dose. Against the background provided by the above notions, the model development approach was as follows:

1. Use allometric scaling to predict the pharmacokinetics in rhesus monkeys based on pharmacokinetic data obtained in rats, cynomolgus monkeys, and dogs.
2. Use the protein binding information in cynomolgus monkeys to estimate the unbound drug concentration in rhesus monkeys.
3. Develop a free drug concentration-behavioral pharmacology (pharmacokinetic–pharmacodynamic) model in rodents. Unfortunately, the exact nature of this CNS test was not reported in either manuscript.
4. Predict the pharmacodynamic response in rhesus monkeys using allometrically scaled unbound drug pharmacokinetics (steps 1 and 2) and the pharmacodynamic model in rodents (step 3), adjusting for the expected differences in receptor binding affinity between rodents and rhesus monkeys.

5. Compare the observed and predicted pharmacodynamic response in rhesus monkeys to validate the underlying pharmacodynamic model. This involves predicting the pharmacodynamic response in humans using the following:
 - a. unbound pharmacokinetic parameters estimated from allometric scaling of rat, cynomolgus monkey, and dog pharmacokinetic and protein binding data, and
 - b. the pharmacokinetic–pharmacodynamic model obtained in rodents adjusted for the differences in receptor binding affinity between rhesus monkeys and humans.
6. Optimize the pharmacodynamic response in humans using Monte Carlo simulation to identify those doses that meet the target criteria.

In vitro receptor binding data indicated that humans and rhesus monkeys have 20-times and 40-times less receptor binding affinity than rodents, respectively. The behavioral effect in rodents was best characterized using a sigmoid $E_{\max}$ model with $E_{\max}$ fixed to 100% maximal effect, $EC_{50} = 0.0238$ ng/mL, and the shape parameter equal to 0.95. After adjusting the potency from rodent to rhesus monkeys based on in vitro receptor binding, i.e., by multiplying EC_{50} by 40, receptor binding in rhesus monkeys was also well characterized (Fig. 3), thus validating the link between pharmacokinetics and receptor occupancy and receptor occupancy and pharmacodynamics.

Having validated the pharmacodynamic model, the authors moved onto finding a dose range for the FTIM study. One common method to choose the maximal dose in an FTIM study is some fraction of the highest dose that produces no observable toxic effects, called the no observable effect level (NOEL), in the most sensitive animal species (see Chapters 2 and 10). Another method is to choose a dose based on exposure, usually area under the curve at steady-state ($AUC_{0-\tau}$), since exposure may better correlate to toxicity than dose. Using the former method, a dose ranging study from 1 to 60 mg was chosen based on 1/3 the NOEL from the one month toxicology study in dogs (5 mg/kg) and 1/5 the NOEL in rats (3 mg/kg). Monte Carlo simulation was then done to predict the exposure in humans relative to the exposure observed in rats and dogs as part of the toxicokinetic evaluation from those studies. Because inter-individual variability was unknown, as was the oral bioavailability and absorption rate constant in humans, several scenarios were evaluated:

1. Oral bioavailability was fixed at 40%, 60%, or 80%.
2. Inter-individual variability on all the pharmacokinetic parameters was assumed to be log-normal in distribution with coefficient of variation 20%, 30%, or 40%.
3. Absorption was first-order fixed at 0.2, 0.5, or 0.8 per hr.

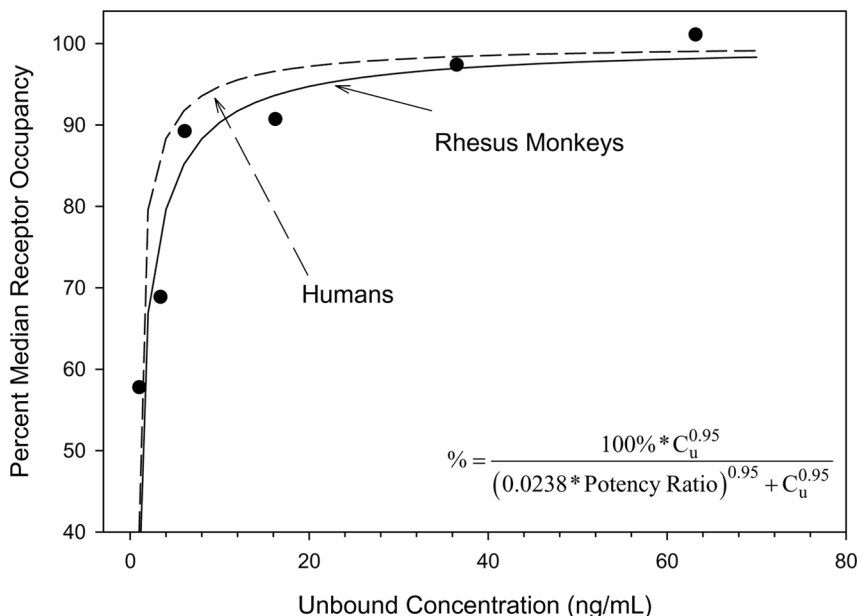


Figure 3 Observed and predicted (solid line) median brain receptor occupancy in two rhesus monkeys as reported by Gomeni et al. (12). Also shown is the predicted occupancy in humans. Predicted occupancy was based on the occupancy model (Sigmoid E_{max}) developed in rodents with EC_{50} adjusted for the differences in affinity between rodents and rhesus monkeys or humans. Maximal binding was assumed to be equal to 100%. Rhesus monkeys have 40-fold lower affinity for the receptor than rodents, whereas humans have 20-fold lower affinity. (Reprinted with permission from Ref. 12. Gomeni et al. (12) Copyright 2001, Drug Information Association.)

The drug's pharmacokinetics were assumed to follow a two-compartment model with first-order absorption. Monte Carlo simulation was then used to simulate pharmacokinetic profiles after single dose administration. Maximal concentration (C_{max}) and AUC were estimated for each subject and the population median and 95th percentile determined. Using the most conservative assumptions (80% bioavailability, high inter-individual variability of 40%, and rapid absorption of 0.8 per hr) at the highest dose studied (60 mg), the ratio of $AUC_{0-\tau}$ in the rat to the simulated 95th percentile for $AUC_{0-\infty}$ in humans (called a coverage factor) was 1.1. In the dog, the AUC cover factor was 1.6. Hence, based on either exposure or factors of the NOEL dose, both methods indicated that the top dose of 60 mg was an appropriate maximal single dose in humans.

Armed with the above information, the investigators developed a novel set of simulations aimed at determining how receptor occupancy

behaved in a multiple dose setting with doses of 10, 30, and 60 mg once daily. With reference to other drugs from the same family, it was previously demonstrated that receptor occupancy greater than 70% during 24 hr chronic treatment maintained over a period of several weeks is efficacious clinically. Hence, Monte Carlo simulation was used to identify dosing regimens that would achieve at least 70% receptor occupancy throughout the steady-state dosing period in the majority of subjects. Predose concentrations at steady-state were used as the target variable since this represents the lowest concentration achieved by a drug once steady-state is achieved. If at least 70% occupancy is achieved at predose, then at least this degree of occupancy will be maintained during the dosing interval immediately after a dose is taken.

The same uncertainties in the single dose simulations still apply with this set of simulations, but with one additional problem: how does the uncertainty in the receptor binding potency in humans affect the results? Hence, such uncertainty needed to be taken into account, and an additional scenario was examined. Three potency values were compared: (a) equal to results of the *in vitro* receptor binding study, (b) equal to the potency in rodents, or (c) equal to the average of (a) and (b). Using the most conservative assumptions (low potency equal to the results of the *in vitro* receptor binding study, low bioavailability of 40%, high inter-individual variability of 40%, and slow absorption of 0.2 per hr) at the highest dose studied (60 mg), 95% of subjects had predose receptor binding occupancy very close (66.1%) to the target of 70%. Less pessimistic assumptions (intermediate potency equal to the average of the rodent and the *in vitro* binding study, intermediate bioavailability of 60%) at the 30 mg dose lead to 95% of subjects attaining predose receptor occupancies of 71%. Hence, based on these results, the authors concluded that the proof of concept study should use a dose ranging study from 30 to 60 mg once daily for a week. However, they also recommended that before such a study is conducted, a positron emission tomography study in humans be done to better characterize the pharmacodynamic model in humans. Regrettably, the authors did not report how well their models actually predicted the results in humans, but the authors, in a personal communication, indicated that their predictions were in full agreement with the observed data. Due to the nature of the drug and the confidentiality policy surrounding this novel class of compounds, they are currently unable to publish their results.

This example illustrates how modeling and simulation may be used to help guide early clinical development of the drug. Drug development has historically based many critical decisions on empirical rules of thumb, such as the starting dose for a single-dose FTIM in man study. According to the historical approach, once the maximal tolerated single dose (MTD) in humans became available, some fraction of the MTD was used as a starting

dose in multiple-dose studies. When tolerability of the multiple dose study was established, usually in healthy volunteers without the disease of interest, the tolerability in patients having the disease was estimated relative to the efficacious dose used in the preclinical studies. Finally, this dose, and maybe one or two others, was taken to phase 2.

Preclinical pharmacokinetic–pharmacodynamic modeling aims to change the historical approach by making the decisions less empirical and more rational. Granted, the approach used by Gomeni et al. may seem like a house of cards, but a modeling and simulation approach has a solid quantitative basis, allows one to test the conclusions for robustness to only partially supported or unsupported hypotheses, and ultimately is still better than empiricism alone. The modeling and simulation approach requires the analyst to identify what is known and unknown about the drug and then evaluate the impact of those uncertainties (or, hopefully, the most significant ones) on the expected outcome. Hopefully, in the end, a more scientific clinical development strategy will make the drug less likely to fail at later, more expensive stages of development.

4.3. Case Study 3: Comparison of Pharmacodynamics in Animals and Humans

Ferron et al. (14) reported the results of a pharmacokinetic–pharmacodynamic model of pantoprazole, an irreversible proton pump inhibitor for the treatment of reflux esophagitis, peptic ulcers, and other acid-related hypersecretory gastrointestinal disorders. In preclinical studies, a stomach catheter was inserted into female Sprague–Dawley rats and the acid content of the effluate was measured at 15 min intervals. Pantoprazole (0.12, 0.23, 0.38, or 1.15 mg/kg) or saline was administered by an intravenous bolus 1 hr after commencement of a 4.5 hr continuous infusion of 1 $\mu\text{g}/\text{min}/\text{kg}$ pentagastrin, a drug that maximally stimulates gastric acid secretion. In a separate group of rats, the pharmacokinetics of pantoprazole were characterized after intravenous infusion of 5 mg/kg over 1 min.

In the clinical studies used to bridge the preclinical results to the human results, pantoprazole pharmacokinetics and pharmacodynamics were studied in humans in three different studies. In the first pharmacokinetic study, healthy male volunteers were randomized in a four-period cross-over study to receive either 10, 20, 40, or 80 mg pantoprazole as a 15 min intravenous infusion, while in a second similar study, healthy male subjects were randomized to receive either 10, 20, 40, or 80 mg enteric coated tablets. In both studies, serial blood samples for drug concentration analysis were collected for 24 hr. In a separate pharmacodynamic study, healthy volunteers who were *H. pylori* negative were administered pentagastrin 1 $\mu\text{g}/\text{kg}/\text{hr}$ for 25 hr. Using a cross-over design, subjects were randomized to receive either placebo, a single dose of pantoprazole

(20, 40, 80, or 120 mg) infused over 15 min, or a single oral dose of pantoprazole 40 mg as an enteric coated tablet. Gastric aspirates were collected by a nasogastric tube every 15 min for 2 hr and every 30 min thereafter until the end of study. The acid content of the gastric contents was measured using titration.

The pharmacokinetics of pantoprazole were characterized using a one-compartment model in rats and a two-compartment model in humans. To account for the oral administration of an enteric coated tablet, a lag compartment was used. Mean concentrations at each dose group were determined and the pharmacokinetic model fit to the data simultaneously for all dose groups. An indirect, irreversible pharmacodynamic response model was used to characterize the pharmacodynamics of pantoprazole in both animals and humans. Since a plot of pH vs. time in the placebo group was essentially constant, the rate of acid output in the effluate (R) was described by

$$\frac{dR}{dt} = K_{\text{prod}} - K_{\text{deg}}R \quad (3)$$

where K_{prod} is the zero-order rate of acid production in the absence of drug (with units mass/hour) and K_{deg} is the endogenous degradation rate of acid (with units per hr). At steady state

$$\frac{dR}{dt} = 0 \quad (4)$$

and $K_{\text{prod}} = K_{\text{deg}} \times R_{\text{ss}}$ where R_{ss} is the basal rate of acid production. In the presence of pantoprazole, an irreversible loss to R is added to the model

$$\frac{dR}{dt} = K_{\text{prod}} - K_{\text{deg}}R - k \times R \times C_p \quad (5)$$

where k is the rate of apparent reaction constant of pantoprazole with the proton pump and C_p is the plasma pantoprazole concentration.

The mean pharmacokinetic parameters were used as inputs to the pharmacodynamic model and the pharmacodynamic model parameters were estimated. The model was able to characterize the pharmacokinetic and pharmacodynamic endpoints across all doses studied (Fig. 4) and was able to predict the rate of acid output after oral administration. The apparent reaction rate between pantoprazole and the proton pump was similar between species (0.691 L/mg/hr for rats and 0.751 L/mg/hr for humans) as was the basal rate of acid output (0.44 mmol/hr/kg for rats and 0.33 mm/hr/kg for humans).

Using the pharmacokinetic and pharmacodynamic model thus developed and the corresponding parameter estimates, the pharmacokinetic and pharmacodynamic profile after oral administration of the 40 mg enteric-coated tablet was simulated and compared to observed data for

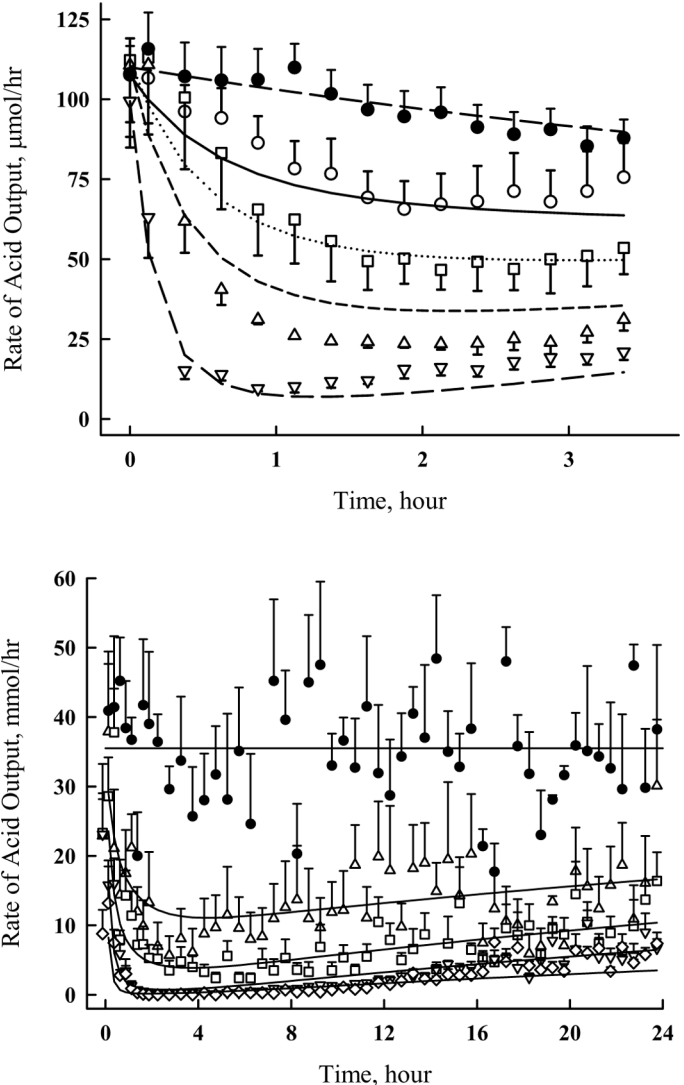


Figure 4 Mean profiles of rate of acid output in rats (top) and humans (bottom) after intravenous administration of pantoprazole following pentagastrin acid stimulation as reported by Ferron et al. (14). Top Legend: solid circle, placebo; open circle, 12 mg/kg; open square, 0.23 mg/kg; open triangle, 0.38 mg/kg; open upside-down triangle, 1.15 mg/kg; open diamond, 5 mg/kg. Bottom legend: solid circle, placebo; open circle, 10 mg; open triangle, 20 mg; open square, 40 mg; open upside-down triangle, 80 mg; open diamond, 120 mg. Figure courtesy of Philip Mayer, Wyeth Laboratories.

validation. The authors then used computer simulation to evaluate the effect of single vs. multiple intravenous and oral doses of pantoprazole 10 to 120 mg and intravenous infusions of 80 mg with infusion lengths varying from 0.5 to 12 hr. The simulations showed that acid output is related to extent of exposure. Acid inhibition increased and remained inhibited longer as dose was increased.

Using the pharmacokinetic and pharmacodynamic model parameters reported by Ferron et al. (14), we simulated plasma concentration and acid output profiles for once-daily dosing of 10, 25, 40, and 55 mg pantoprazole. The results are shown in Fig. 5. Despite no accumulation of pantoprazole even at the highest dose, repeated administration suppressed acid output after a few days of dosing, even at the lowest dose. The difference between the doses was largely the time to maximal suppression. Increasing doses resulted in a decrease in the time to maximal suppression, to a point. A difference between 10 and 25 mg was apparent, as was a difference between 25 and 40 mg, but there was little difference between 40 and 55 mg. Increasing the dose beyond 40 mg appeared to offer little benefit. Interestingly, pantoprazole is currently marketed (Protonix®) as either a 40 or 20 mg enteric-coated tablet.

Now, for the sake of argument, let us suppose that pantoprazole did not make it to market. The authors would have now developed a useful pharmacokinetic–pharmacodynamic model that could be used to help develop back-up pipeline compounds. If the discovery chemists were able to synthesize a series of compounds presumably able to inhibit proton pumps, the animal model could be used as a screen to help choose an appropriate back-up compound, as well as aid in dose selection for the first time in man studies. The rat model could also be used to study drug–drug interactions, the effect of food, or any other relevant scientific question deemed of importance, and would tie these results directly to the pharmacodynamic effects in humans.

4.4. Case Study 4: Integrating In Vitro Methodologies into Antimicrobial Drug Development

Traditionally, preclinical studies are by default performed in animals. With the advances in cell culture, molecular biology, and other in vitro methodologies, the traditional use of the word “preclinical” is rapidly becoming too narrowly defined. Today, preclinical investigation needs to be more inclusive and may mean any model system not including humans. With that in mind, Drusano et al. (15) have reported a useful application of pharmacokinetic–pharmacodynamic modeling and simulation using antimicrobial sensitivity in isolates (aseptically collected specimens from lesions or sputum from patients with the pathogen) from patients infected with a particular pathogen. The first reported use was with evernimicin, the first member of a

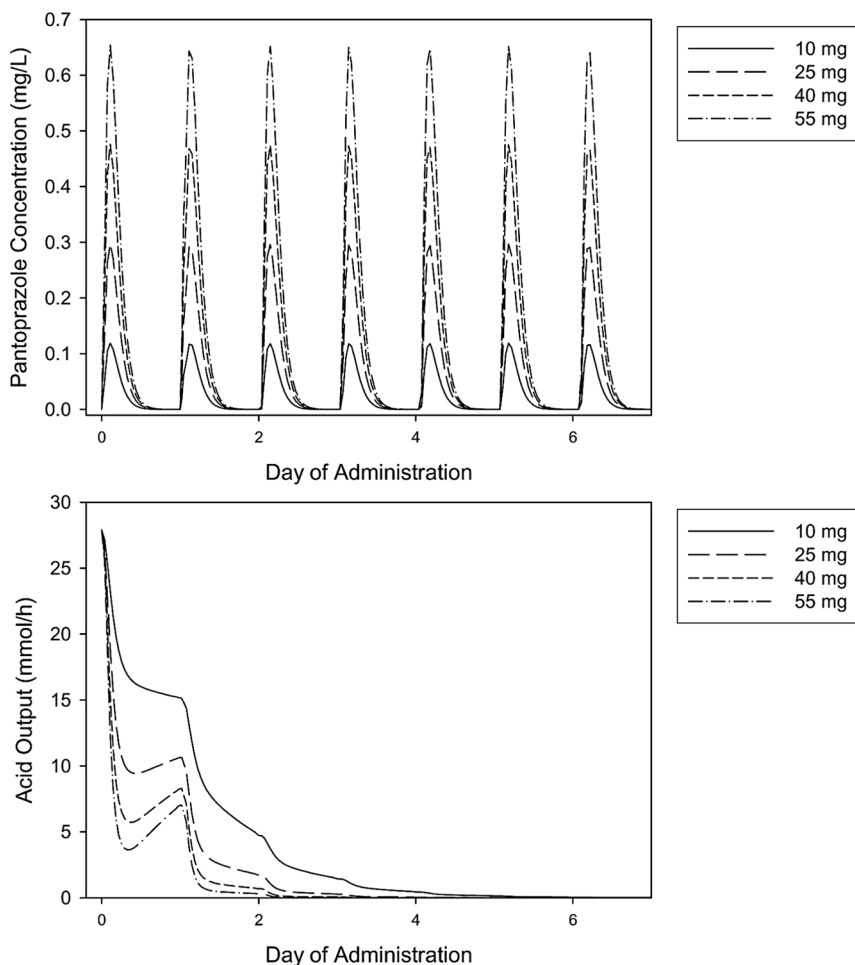


Figure 5 Simulated plasma concentration (top) and acid output (bottom) profiles in humans dosed once-daily with 10, 25, 40, or 55 mg enteric-coated pantoprazole using the model and parameter values reported by Ferron et al. (14).

unique class of oligosaccharide antibiotics active against gram-positive organisms that was later discontinued from clinical development because the drug failed to show a better activity and safety profile compared to already marketed agents (15). The basic idea is one that has been used often before: utilize preclinical data to obtain some measure of clinical exposure needed for activity, apply population pharmacokinetics to understand the pharmacokinetic behavior of the drug in humans, then use Monte Carlo simulation to vary the dosing regimen until some percentage of simulated patients obtain the target preclinical outcome.

Antibiotics are classified into two broad classes: bactericidal agents, which kill the organisms by interfering with cell wall synthesis or some other key metabolic function of the microbe, and bacteristatic agents, which inhibit the growth of the organism. The drug concentration that inhibits bacterial growth for 24 hr is called the minimum inhibitory concentration (MIC) and the concentration that inhibits such growth in X% of isolates is called the MIC_X. For example, the concentration that inhibits 80% growth is called the MIC₈₀. Not all organisms are killed in the same manner by bactericidal drugs. Some drugs kill in a concentration-dependent manner, e.g., aminoglycosides, and the higher the blood drug concentration or area under the curve (AUC) relative to the MIC the more effective the drug. For other drugs, it is not the actual drug concentration that is important, but how long the drug's concentration remains above the MIC, e.g., macrolides and β -lactams. Thus, microbiologists investigate which of three possible parameters relates better to outcome: the ratio of AUC to MIC (AUC/MIC ratio), the ratio of peak antibiotic concentration to MIC ratio (peak/MIC ratio), and the percent of time above the MIC. Which of these three parameters is important for predicting response is drug-dependent.

Drusano et al. (15) used a murine model of infection and studied three pharmacodynamic endpoints: stasis (that value which resulted in no change in the number of bacteria beyond the colony forming unit at the time of inoculation), log killing (calculated from the modeled maximal colony count in the control group), and 90% $E_{\max}$ (calculated as the log drop representing 90% of the maximal log drop achievable). The independent variables examined were AUC/MIC ratio, peak/MIC ratio, and percent time above the MIC. Separately, the MICs for each of about 1500 isolates were determined and the MIC₈₀ against pneumococci, staphylococci, and enterococci was estimated. Next, the protein binding of evernimicin in mouse and human plasma was estimated. Then, the pharmacokinetics of evernimicin in healthy normal volunteers and in patients with hepatic impairment was characterized using population pharmacokinetics approaches. Lastly, the distribution of MICs and pharmacokinetics of evernimicin were simulated under two dosing regimens. The percent of subjects meeting the *in vivo* targets from the murine mouse model was finally determined. Fig. 6 illustrates the process.

The murine model showed that all three independent variables predicted outcome about equally well with AUC/MIC ratio being slightly better than the other two predictors (Fig. 7). As also mentioned earlier, since unbound drug is important for pharmacodynamic activity, any model for antimicrobial pharmacodynamics needs to use unbound concentration as the independent variable. However, in this case, there was no species difference in degree of binding. So, based on this observation and to simplify matters, the authors used total drug concentration, instead of free drug concentration, as the independent variable in future simulations. Subsequently,

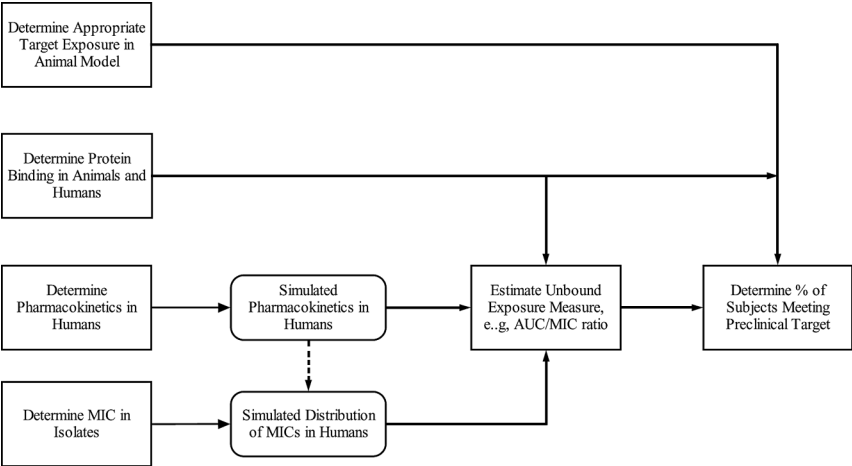


Figure 6 Schematic model used by Drusano et al. (15) in the Monte Carlo simulation of antimicrobial dosing regimens.

using two dosing regimens, 6 mg/kg and 9 mg/kg evernimicin once daily, the percent of subjects attaining the preclinical target was determined. Table 1 shows the percent of subjects reaching the preclinical targets for each of the pathogens studied. The simulations showed that the lowest dose provided sufficient exposure near the top of the dose–response curve against all three pathogen categories. Also, evernimicin is such a potent drug that

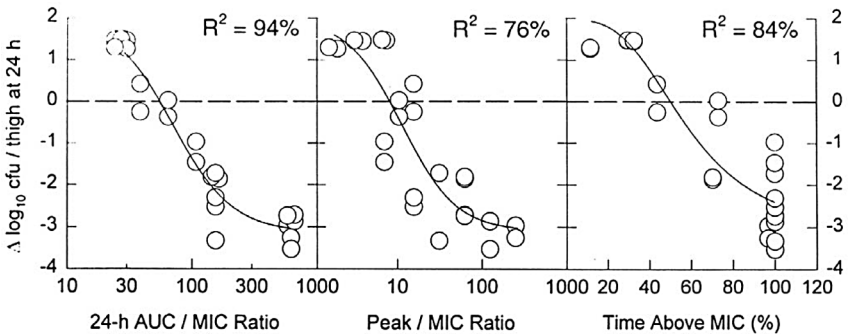


Figure 7 Change in colony forming units (cfu) recovered from mouse thigh model of infection at 24 hr after initiation of therapy with evernimicin as a function of AUC/MIC ratio, peak/MIC ratio, and percent time above the MIC. The AUC/MIC ratio gives slightly better predictability of outcome compared to the other measures. (Reprinted with permission from Ref. 15. Courtesy of the American Society for Microbiology.)

Table 1 Percent of Subjects Reaching Evernimicin Preclinical Targets

Dose (mg/kg)	<i>S. pneumonia</i>			<i>S. aureus</i>			Enterococci		
	Stasis	Log drop	90% <i>E</i> _{max}	Stasis	Log drop	90% <i>E</i> _{max}	Stasis	Log drop	90% <i>E</i> _{max}
6	100	100	96	92	72	34	100	100	58
9	100	100	98	97	85	50	100	100	79

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a 50% increase in dose resulted in little change in the number of subjects reaching the preclinical targets.

A second application of this methodology was reported the next year with GW 420867X, a non-nucleoside reverse transcriptase inhibitor of human immunodeficiency virus type 1 (HIV-1) (16). In this study, the authors used two preclinical pieces of information: in vitro protein binding of the drug in human plasma and the distribution of concentrations that inhibit 90% of viral growth (*EC*₉₀) to test HIV isolates. The pharmacokinetics of GW420867X was then characterized using nonparametric population-based methods with data obtained from a multiple-dose study in normal healthy volunteers. Assuming the pharmacokinetics in healthy volunteers will be reflective of the pharmacokinetics in patients with HIV and that time above the *EC*₉₀ will be the important pharmacodynamic target, the clinical information was then combined with the preclinical information to create a joint model predicting unbound drug concentrations in patients at steady state. Using Monte Carlo simulation, the authors tested three dosing regimens (50, 100, and 200 mg once daily) to determine the percent of subjects with simulated unbound trough drug concentrations greater than 10-times the *EC*₉₀ and *EC*₅₀. Based on the simulation, each of the doses provided >95% target attainment when the *EC*₅₀ was less than 10 nM. At the time of publication, the authors indicated that, among the 16 isolates available, all had *EC*₅₀s less than 8 nM. In summary, by combining relevant preclinical targets with clinical information, Drusano et al. were able to either predict a relevant dosing regimen or to make conclusions about differences in already selected dosing regimens.

5. CONCLUSIONS

Preclinical pharmacokinetic–pharmacodynamic modeling has most value early in drug development, prior to Phase 2, when the clinically useful dosing regimen is still undefined. At this early stage, the pharmacokinetics in humans can be estimated using allometric scaling (Chapter 2). Then, given

a pharmacodynamic model in animals, the pharmacokinetic–pharmacodynamic profile in humans can be predicted. Once the first time in man study (single dose and/or multiple dose) is performed, a more complete pharmacokinetic–pharmacodynamic model can be developed. Hence, in a rationally designed drug development program, the target dosing regimen is based on integrating preclinical and clinical information into a cohesive quantitative model. When the relationship between dose and concentration in humans and the relationship between concentration and pharmacodynamics in animals are established, more relevant dosing regimens can be designed and optimized for Phase 2 trials. After Phase 2, the value of preclinical pharmacokinetic–pharmacodynamic models decreases, except to aid in identifying and characterizing back-up drugs, because the focus then shifts towards developing a clinical pharmacokinetic–pharmacodynamic model, a more relevant goal oriented towards the final product, a newly marketed drug for the company.

Lastly, a disclaimer. The problem with reviews, such as this chapter, is that afterwards there is an implicit expectation that effort devoted to modeling and simulation will lead to useful models. All of the examples in this chapter represent publication bias in that the results were novel or useful in helping guide drug development and are therefore, “publishable.” In reality, modeling efforts that fail, ones where a suitable model could not be developed or ones where the model did not predict effects accurately in human, tend not to get published. Thus, the impression is made that modeling and simulation is universally useful and that failure to find a successful model then reflects as some failure on the analyst. Ironically, it is probably these failed models that are most informative since understanding under what conditions a model may fail may be more useful to a pharmacokineticist than yet another application of a linear or $E_{\max}$ model to model the pharmacodynamics of a new drug. Before unrealistic expectations are placed on the pharmacokineticist, it is important to emphasize that not all modeling is successful. Companies (i.e., managers, project team leaders, clinicians, etc.) need to understand that some modeling efforts will be wasted or may point development in the wrong direction. However, when a model does succeed and is useful in helping guide drug development, the rewards to the company and perhaps science as a whole can be substantial.

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Formulation and Route of Administration—Influencing Drug Permeability and Absorption

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Drug efficacy, and to some degree minimization of toxicity, is predicated on appropriate formulation and delivery to the required site of action via the systemic circulation. A drug delivery system that contains a drug or a combination of drugs with excipients is formulated in such a way that it releases drugs in a desired manner at the required site. The performance of a drug delivery system depends upon the nature and amount of excipients used, method of preparation, and the route of administration. The drug delivery system is required to be safe, effective, and reliable for an optimum therapeutic response. An important consideration for the delivery of drugs to systemic circulation is its route of entry in the body. A drug can be administered by non-invasive routes such as oral, transdermal, ocular, nasal, pulmonary, vaginal, and rectal routes, or by invasive routes such as implants or injections that are intravenous, intramuscular, subcutaneous, intradermal, and intrathecal. This chapter is intended to provide an introduction to the challenges associated with the development and preclinical evaluation

of delivery systems via the most common non-invasive routes of drug administration.

1. ORAL DRUG DELIVERY

Oral administration of drugs by conventional or novel pharmaceutical formulations is the most convenient and effective delivery system and is preferred over parenteral delivery of medication. However, not all drugs can be administered orally, mainly because of their instability in gastric acid, vulnerability to gastrointestinal enzymes, first pass metabolism, and low oral permeability. The site-specific delivery of a drug to its target receptor site has the potential to reduce side effects and to increase pharmacological response. In addition, there are a number of local pathologies where direct release of drug in a specific site along the gastrointestinal (GI) tract would not only improve pharmacotherapy but also would reduce potential toxicity and side effects. The treatment of disorders of the GI tract, such as irritable bowel syndrome, colitis, Crohn's disease, colon cancer and local infectious diseases where a high concentration of active agent is needed, can be markedly improved using site-specific delivery systems employing various designs.

1.1. Anatomy and Physiology of the GI Tract

A brief review of GI anatomy and physiology should provide some insight into the design of oral drug delivery systems. The GI tract can be divided into four sections based on the organs of digestion along the alimentary canal.

1.1.1. Oral Cavity

The first section of the alimentary canal that a drug comes in contact with is the oral cavity. Seldom does the dosage form remain in this cavity long enough for drug absorption to take place, unless the drug is administered buccally or sublingually. The use of a buccal bioadhesive device in targeting controlled drug delivery to the gastrointestinal tract has been investigated (1).

Usually, an orally administered dosage form (i.e., tablet, capsule, solution, or suspension) quickly reaches the stomach after traveling down the esophagus. Drug absorption does not normally occur in the esophagus because the transport rate of the dosage form through the esophagus into the stomach far exceeds the rate of drug liberation from the dosage form or absorption of drug across the esophagus. However, an exception to this rule is the engineered formulation of magnetic granules consisting of bleomycin (an anticancer drug), bioadhesive polymers and ultrafine ferrite were prepared and evaluated for targeted drug delivery to the esophagus in an animal model of esophageal cancer (2).

1.1.2. Stomach

The adult human stomach has a capacity of 1.5 L. The contents of the stomach include hydrochloric acid, pepsinogen, and mucus. The pH of the stomach in a normal, healthy human can range from 1 to 3. The time period for oral drug delivery completion is unpredictable and may be very short in certain circumstances. Accordingly, drug absorption in the GI tract may be highly variable. Thus, there should be a major interest to develop sustained-release delivery systems that can be retained in the stomach for a prolonged and predictable period of time. Targeted drug release in the stomach is attractive for several reasons: (1) better dissolution of weak bases that are poorly soluble at higher pH; (2) any solute released in the stomach will empty together with GI fluids and will have the whole surface of the intestine for absorption. This should be particularly useful for drugs where an absorption window exists in the proximal regions of the small intestine (e.g., riboflavin, levodopa); (3) gastric release also appears to be useful for all substances intended to produce a sustained effect locally at the gastroduodenal wall. Although attractive, targeting of drugs to stomach is not recommended for poorly acid-soluble drugs, drugs unstable in the gastric environment, drugs that are irritating to the gastric mucosa, enteric coated systems, and drugs intended for selective delivery to the colon.

1.1.3. Small Intestine

The first distinct section of small intestine is the duodenum. The pH of the duodenum is approximately 6. In addition to water, mucus, and electrolytes, bile from the liver and secretion from the pancreas join secretions from the intestinal mucosa to facilitate digestion and absorption. The small intestine is the primary site of absorption in the GI tract, as it possesses an enormously large surface area. The lining of the lower part of small intestines is composed of many villi, or finger like projections, which extend even more as projections called the brush border. This area is highly perfused with blood and is a predominant region of absorption for most drugs. The pH in the jejunum region is between 7 and 8.

As an example of drug absorption, amoxicillin was found to be well absorbed in the duodenum and jejunum, less so in a rate-dependent manner in the ileum, and was not absorbed when infused in all colonic regions (3). As discussed in [Chapter 8](#), a wide variety of transporters are expressed in the small intestine, and these transporters are involved in the membrane uptake of daily nutrients as well as drugs (4). These transporters are located in the brush border membrane as well as basolateral membrane. Each transporter exhibits its own substrate specificity, and some have broader specificities than others. In addition, the expression and characteristics of the intestinal transporters exhibit regional differences along the intestine, implying diverse physiologic functions and, in some cases, pathologic responses. The development

of prodrugs that target intestinal transporters has been successful in improving oral drug absorption. For example, the intestinal peptide transporter is utilized in order to increase the bioavailability of several classes of peptidomimetic drugs, especially ACE inhibitors and beta-lactam antibiotics. The bioavailability of poorly absorbed drugs can be improved by utilization of the transporters responsible for the intestinal absorption of various solutes. Alternatively, inhibition of membrane transporters involved in drug efflux such as p-glycoprotein (Pgp) can also increase bioavailability.

1.1.4. Large Intestine

The large intestine includes the colon and rectum. In contrast to the small intestine, the colon does not contain villi. In fact, the surface of the colon is essentially flat but contains numerous crypts. The colon contains high concentrations of bacteria (as many as 10^{12} per mL) that serve various functions. The pH in the colon region ranges from 7 to 8. Oral colon-specific drug delivery systems have become increasingly popular because they offer obvious advantages over parenteral administration. Colonic targeting is naturally of value for the local treatment of diseases of the colon such as Crohn's disease, ulcerative colitis, and colorectal cancer (5).

1.1.5. Wall of the Digestive Tract

The inner region of the digestive tract is termed the mucosal surface. It consists of a surface layer of cells, called the epithelium. Under the epithelium is loose connective tissue that has an extensive blood supply and also contains an active population of immune-related cells. Several mucosa-adhesive systems have been studied. Initial *in vivo* studies indicated that the incorporation of carbomer 974P (Carbopol 974P) into a delivery system helped improve the bioavailability of insulin. Absorption of drugs and vaccines at mucosal surfaces may be enhanced by conjugation to appropriate bioadhesives that bind to mucosal epithelia (6). Recent studies have indicated that a number of polymeric delivery systems possess significant potential for the development of mucosally administered vaccines (7).

Below the mucosa is the submucosa layer, which consists of dense connective tissue, larger blood vessels, and nerves that supply the various glands in the mucosa. The third layer is the muscularis, which provides shape and flexibility to the GI tract. The last or the outermost layer forms the serosa.

1.2. Factors Affecting the Oral Absorption

Oral drug absorption takes place by passive, ionic, active, facilitated, transcytosis, and lymphatic pathways (8). One should be careful about the loss or reduction of absorption due to luminal or epithelial degradation, and efflux transporters such as P-gp. Several factors contribute to

an increase or decrease of drug absorption. These factors are usually classified as physicochemical factors, physiological factors, and formulation factors. Physicochemical factors include solubility, particle size and distribution, crystal habit and polymorphism, drug pK_a , $\log P$, and stability. Among the important physiological factors are the site of absorption, gastrointestinal blood flow, pH, gastric emptying, presence and absence of food. Formulation factors include the nature of dosage form, excipient selection and process variables, and routes of administration. There are several texts and review articles on this topic (9). It is important to realize that for most drugs, permeability and absorption through the GI tracts are not uniform. As an example, recent studies in our laboratory have shown that ubiquinone (a lipophilic molecule) permeability in rat GI tract is greater for duodenal segments as compared to that of other regions. This is demonstrated in Figure 1. On the other hand, the permeability of insulin was found to be greater in the jejunum and ileum areas as compared to others (10).

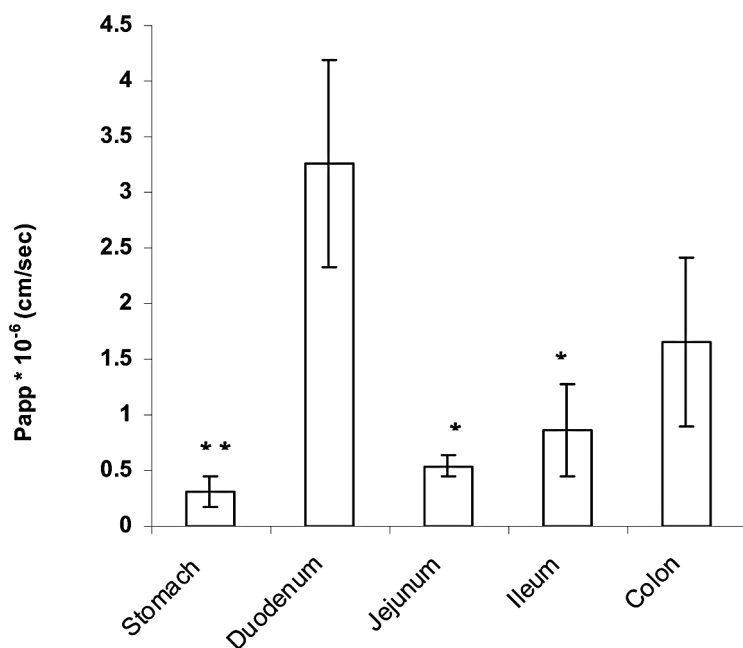


Figure 1 Regional permeability of ubiquinone across various gastrointestinal segments of rats (n equals 6).

For more challenging drugs such as proteins and peptides, oral delivery is severely limited by enzymatic degradation, low permeability because of high molecular weight, and formulation challenges. As a rule of thumb, if the molecular weight is more than 500 then permeability problems can be anticipated. However, some problems not associated with molecular weight can be overcome by a careful selection of enzyme inhibitors and/or permeability enhancers (11), and appropriate selection of manufacturing techniques (12). Recent studies in our laboratory have indicated that salmon calcitonin degradation due to trypsin, α -chymotrypsin, and elastase enzymes could be easily overcome by appropriate concentrations of ovomucoids (13). As shown in Figs. 2 and 3, salmon calcitonin protection by the enzyme inhibitory activity of ovomucoids was species as well as concentration dependent. The enzyme degradation has led to an overall increase in permeability. While the formulation and bioavailability studies are in progress, similar studies with insulin have apparent enhanced bioavailability and reduced glucose levels with a dual controlled release formulation (12). In this formulation, both insulin and ovomucoid were released in a controlled fashion with a targeted delivery to small intestine.

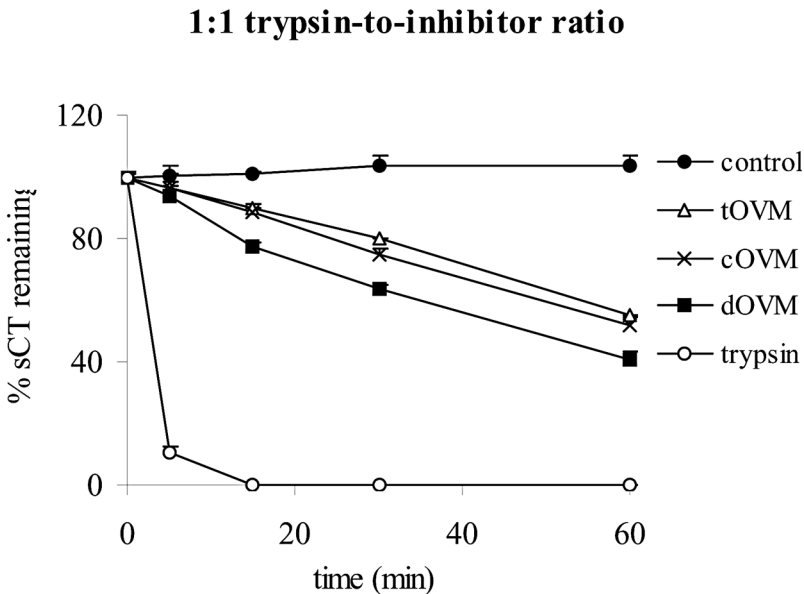


Figure 2 Salmon calcitonin degradation by trypsin and its protection by chicken, duck, and turkey ovomucoids.

Chymotrypsin:turkey ovomucoid

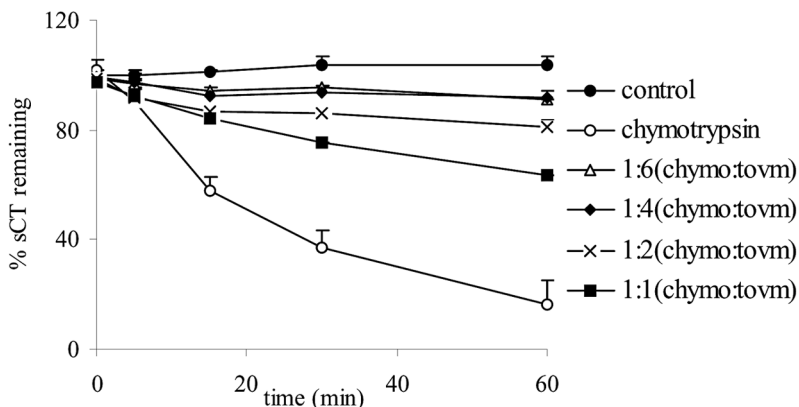


Figure 3 Salmon calcitonin degradation by α -Chymotrypsin and its protection by turkey ovomucoids in different enzyme:inhibitor ratios.

1.3. Experimental Models for Oral Transport Studies

Intestinal transport and metabolism can be studied using *in vitro*, *in situ*, and *in vivo* techniques. An extensive summary of details of various methods can be found elsewhere (14,15).

1.3.1. *In vitro* Methods

Everted intestinal rings (slices): The intestine of the animal is cut into rings or slices and placed in an incubation media that is agitated and oxygenated. These studies are usually conducted for a short period of time. Samples of incubation media and rings are analyzed for drug content. This method has been used for analysis of passive (16) and carrier-mediated transport (17).

Everted intestinal sac: In this technique, a small segment of the intestine (2–3 cm) is sealed (tied) at each end after eversion on a glass rod. The mucosa then faces the outer buffer solution and the serosa becomes the inside of the sac. An oxygenated buffer solution is injected into a sac, which is then immersed into a beaker that contains a drug solution. Samples are collected from the buffer solution and analyzed. This method has been used to determine intestinal permeability of numerous compounds.

Brush border membrane vesicles (BBMV): This method is based on the homogenization of an everted frozen intestine to give a purified fraction of the apical cell membranes from the chosen region of the GIT. This

technique is frequently used for isolated studies of the brush-border membrane transport characteristics.

Cell culture techniques: The use of Caco-2 cell monolayers and other cell cultures (HT29, IEC-18) from a human carcinoma cell line has been extremely popular. They consist of a monolayer of polarized cells grown on a filter support. After the cells are fully differentiated, they express the transport characteristics of villus cells. The transport characteristics of drugs are studied in the inserts or by mounting them in side-by-side diffusion cells. Excellent correlations have been obtained between permeability coefficients determined in Caco-2 cells and absorption in humans (18).

1.3.2. In situ Methods

Isolated perfusion or in situ perfusion: In these methods, a 10–30 cm segment of the intestine is cannulated on both ends and perfused with a buffer solution at a low flow rate (0.2 mL/min). The mesenteric vein and artery are cannulated for the purposes of blood collection. Drug absorption is characterized by the differences in perfusate concentrations (input vs. output) or by monitoring the appearance of drug in the plasma.

1.3.3. In vivo Methods

In vivo methods include bioavailability assessments in different animal models including humans, intestinal perfusions in man, and triple lumen perfusions. These studies are performed in preclinical development or in the early clinical phase to obtain a complete understanding of the absorption of a certain drug, for information on the pharmaceutical dosage form program, and correlation of data obtained from simple animal models. There are two general methods used for in vivo animal perfusion studies.

In vivo single pass perfusion: This perfusion is primarily performed in rat intestines, wherein a small portion of intestine (5–6 cm) is isolated with the blood supply intact. The top to bottom portion of the segment is connected. By means of a cannula, drug is perfused through the intestinal segment. A similar connection via a cannula facilitates collection of perfusate solution from the intestinal loop. Details of the experimental design and data analysis procedures can be obtained from Ref. (19).

Loc-I-Gut technique in dog intestine: In this method, a polyvinyl tube is inserted orally and threaded to the upper GI tract of a dog in the standing position. The polyvinyl tube has six inner channels and two latex balloons. Once the tube reaches the proximal jejunum, the two balloons are inflated. Between the two balloons, there are three openings; one for the perfusate to enter and the other two for the perfusate to exit. Perfusate is infused as a single pass at a rate of 2–3 mL/min. The steady-state intestinal permeability (P_{eff} cm/sec) is calculated by the equation. (see Ref. 20):

$$P_{\text{eff}} = \frac{1}{A} Q_{\text{in}} \frac{(C_{\text{in}} - C_{\text{out}})}{C_{\text{in}}}$$

where Q_{in} is the perfusion flow rate, A is the area of the intestinal segment, C_{in} and C_{out} are the inlet and outlet concentrations of the drug in the perfusate.

1.4. Drug Delivery Systems for Oral Use

Drug delivery systems (DDS) are a strategic tool for expanding markets and indications, extending product life cycles, and generating new market opportunities. The reader is encouraged to explore the literature for information on the numerous and diverse drug delivery systems that are available for assessment. Some of these systems improve palatability, while others may improve stability, solubility, or permeability—subsequently improving bioavailability. Other systems can sustain the release of drug from the delivery system and thus reduce the frequency of dose administrations. A brief description of these oral delivery systems can be found elsewhere (21).

2. TRANSDERMAL DRUG DELIVERY

Transdermal drug delivery is used for the administration of drug molecules through the skin into the circulatory system.

The advantages of transdermal over conventional routes include avoidance of the variables associated with gastrointestinal absorption, reduction of first-pass metabolism (deactivation by enzymes in the liver), and improved therapeutic management of disease by the absence of pulsed delivery. The dosage units used to achieve transdermal drug delivery are called transdermal drug delivery systems (TDDS).

TDDS typically produce a constant plasma drug concentration as opposed to the broader peaks and troughs of intermittent dosing. Since first-pass metabolism is bypassed or reduced, the required dosage is in some cases considerably lower than the amount required orally. Also, bypassing the liver can reduce potential risk of hepatotoxicity that would otherwise be higher due to first-pass exposure. The continuous administration provides sustained plasma levels of the drug and improves patient compliance. Further, it provides an easy method for terminating drug delivery by simply removing the patch from the skin. Since better control of release is possible with TDDS, drugs with low therapeutic indices can be formulated for delivery across the skin.

However, there are several disadvantages of TDDS. The main disadvantage is that there are relatively few suitable drug candidates for transdermal delivery. The poor permeability of human skin to most drugs

necessitates application of a device with an unacceptably large surface area. This may further lead to local irritation or sensitization of the skin provoked by contact with the applied drug (or other components of the delivery device). Difficulties of adhesion to different types of skin, size of the patches, and resultant variation in duration of application to the skin are other factors that have to be taken into consideration. Medications possessing the following characteristics are not acceptable candidates for controlled transdermal delivery: (1) large molecular weight compounds which are poorly absorbed through the skin, (2) drugs that are metabolized in the skin before reaching its site of action, and (3) low efficacy/potency drugs that require large doses.

2.1. Structure of the Skin

TDDS involves direct application of drugs onto the skin. Therefore, it is essential when developing a TDDS to understand the physical and chemical composition of the skin, which acts as the main barrier for entry of drugs into circulation. The skin is an easily accessible organ for drug administration. The human skin covers a surface area of approximately 3000 in². Microscopically, the skin is a multi-layered organ composed of histologically distinct structures. A thin, irregular, discontinuous layer of sebum mixed with sweat, bacteria, and dead cells covers the surface of the skin. The approximate thickness of this layer varies from 0.4 to 10 μm (22). Sebum is produced most abundantly on the forehead, less on the trunk, and very little on the extremities. Sebum composition varies with age, sex, anatomical site, and race. Changes in season and climate can affect the rate of production and contents of skin surface lipids. Therefore, it is important that permeability and absorption studies are carried out systematically for consistency of drug availability in the circulation.

2.1.1. Epidermis

The outer layer of skin is composed of stratified squamous epithelial cells. The epithelial cells are held together mainly by highly convoluted interlocking bridges, which are responsible for the unique integrity of the skin. The epidermis is thick in the areas of the palms of the hands and soles of the feet, and becomes thinner over the ventral surface of the trunk. The epidermis consists of two main parts, the stratum corneum and the stratum germinativum. The stratum corneum forms the outermost layer of the epidermis and consists of many layers of compact, flattened, dehydrated, keratinized, hexagonal cells in stratified layers. These are called horny cells and are metabolically inactive. The stratum corneum is responsible for barrier function of the skin, and also behaves as the primary barrier to percutaneous absorption. The thickness of this layer is mainly determined by the extent of stimulation of the skin surface by abrasion and bearing of weight. The stratum

corneum cells are continuously replenished by the regeneration of new cells and migration of cells produced by the basal or basement layer of the epidermis. In the thicker parts of the skin, the transition from the living cells of the germinativum zone to the dead cornified cells of the stratum corneum is made prominent by three layers, the stratum spinosum (prickly layer), stratum granulosum (granular layer), and the stratum lucidum (clear layer). Like the stratum corneum, the stratum granulosum and stratum lucidum are also physiologically important. Removal of these three layers results in water loss and an enhancement of skin permeability (23).

2.1.2. Dermis

The dermis is a complex structure consisting mainly a network of robust collagen fibers of fairly uniform thickness with cross-striations that are regularly spaced. Dermis cells include fibroblasts, mast cells, blood cells, nerve cells, and endothelial cells. The dermis also contains oriented tropocollagen (polypeptide) macromolecules. This network or gel structure is responsible for the elastic properties of the skin. The dermis has a rich network of sensory nerves, blood capillaries, and lymphatics. The upper portion of the dermis is formed into ridges (or papillae) projecting into the epidermis, and contains blood vessels, lymphatics, and nerve endings. A fibrous tissue opens out beneath the dermis and merges with the subcutaneous tissue that contains fat (24).

2.1.3. Subcutaneous Tissue

A sheet of fat containing areolar tissue underlying dermis constitutes the subcutaneous tissue. It is also known as the superficial fascia and attaches dermis to the underlying structures. When a drug is applied topically, the drug molecules reach the vascularized dermal and subcutaneous layers where absorption into the systemic circulation can occur.

2.2. Transport of Drug Molecules Across the Skin

There are three transdermal diffusive routes that drug molecules utilize when applied to the skin:

1. transappendageal route,
2. transcellular route, and
3. paracellular route.

When a drug is applied onto the intact skin in a given vehicle or a delivery system, the drug molecules first come in contact with the sebum, cellular debris, and bacteria on the skin surface, and other exogenous material which covers the skin (25). The actual pathways for transappendageal penetration via the pilosebaceous apparatus could be through the hair follicle itself, through the outer root sheath of the hair into the viable cells of the

follicle, or through the air-filled canal and into the sebaceous gland. The sweat ducts enter through either the lumen or the walls of the epidermis and through a thin ring of keratinized cells. Dense capillary networks closely envelope the bases of both the sweat ducts and hair follicles. So, the molecules reaching this highly vascular network region would immediately diffuse into the systemic circulation. The keratin in the intercellular region presents a network of polar and non-polar regions in which substances dissolve and diffuse according to their chemical affinities (26). Electrolytes and other large molecules with low diffusion coefficients pass mainly through the appendages and the appendageal routes under both transient and the steady-state conditions (27).

The transepidermal route is a term that collectively refers both transcellular and paracellular routes. Percutaneous absorption includes the entire process of mass transport of the drug molecules applied topically. It also includes the absorption of topically applied drug molecules across each layer of the skin, culminating in uptake by microcirculation of the skin. The individual transport events occurring in percutaneous absorption are in the following sequence. First, the drug molecules are absorbed into the surface layers of stratum corneum and then diffuse through the stratum corneum into the viable epidermis. This is followed by diffusion through epidermis and dermis. The final stage in the sequence of percutaneous absorption is the uptake of diffusing drug molecules by the microcirculation for systemic distribution (27).

The overall rate-limiting barrier to percutaneous absorption is the stratum corneum. Because stratum corneum cells are dead, diffusion is a passive process governed by physicochemical laws. No active transport process has been shown to be involved in the skin permeation.

When the drugs are applied to the skin, Fick's first law of diffusion governs the rate of mass transfer or flux across the skin, which is given by

$$J_s = -D \times \frac{dC}{dX}$$

where dc/dx is concentration gradient and D is diffusion coefficient. This equation can be modified as follows:

$$J_s = -\frac{dM/S}{dt} = \frac{DKC_d}{h}$$

where M is the amount permeated, S is the cross-section through which permeation takes place; t is the time, C_d is the concentration of the diffusant in donor compartment, and h is the thickness of the membrane through which the drug passes. In the case of TDDS, the flux values are determined based on the following equation:

$$K_o = -Cl_o \times C_p$$

$$J_s = \frac{K_o}{\text{Size}_{\text{patch}}}$$

where K_o is the target in vivo Drug delivery rate (mg/hr); Cl_o is the total body clearance; C_p is the target steady-state plasma concentration, and J_s is the steady-state skin flux (22).

2.3. Evaluation of Transdermal Drug Delivery Kinetics

To evaluate the efficacy of the TDDS systems, in vitro and in vivo analyses are performed. In vitro studies are then correlated with the in vivo data.

2.3.1. In vitro Drug Release Kinetics

For in vitro studies, human cadaver skin is preferred for the evaluation of drug permeation profiles. Since human cadaver skin is difficult to obtain and very expensive, other alternatives such as hairless mouse skin, guinea pig skin, Epiderm[®], and other bio-engineered human skin equivalents are used. The release and skin permeation kinetics can be evaluated using a two-compartment diffusion cell assembly. This is carried out by individually mounting a skin specimen excised from either a human cadaver or other animal, on a diffusion cell. The experiment involves placing the drug-releasing surface of the transdermal delivery system on the skin in intimate contact with the stratum corneum surface, and mounting this in the diffusion cell assembly. Samples are collected from the receiver solution at predetermined time intervals until a steady-state flux is established. Samples are assayed for drug concentration and the concentration data are modeled for diffusivity characteristics. Published examples of utilizing these technologies can be found in Refs. 28 and 29.

2.3.2. Animal Models

The in vivo transdermal permeability of drugs depends on the animal species from which the skin is obtained. The reader is advised to read the review article by Panchagnula et al. (30). The authors presented the permeability differences of a polar and non-polar drug through the skin of 16 animal species including humans.

3. NASAL DRUG DELIVERY

Over the past two decades, intranasal drug delivery has shown tremendous promise for systemic delivery of therapeutic agents, although the potential of the nose as a route of administration has been known since ancient time. Psychotropic and hallucinogenic agents have been used as snuff in many

parts of the world for hundreds of years. Because of a rich vasculature and highly permeable structure, the nasal route is a useful alternative to parenteral routes of delivery. The nasal route circumvents presystemic metabolism by the liver and small intestine (gut wall enzyme mediated degradation). The nose provides a non-invasive, easily accessible route for self-administration by the consumer. Other advantages of nasal drug delivery systems include a rapid onset of action, reduced risk of overdose, and improved patient compliance. However, there are several disadvantages of nasal administration including impermeability of nasal mucosa to lipophilic and high molecular weight drugs, mucotoxicity associated with long-term use of certain formulations, requirement of an expensive delivery device, and dose inaccuracy.

3.1. Nasal Anatomy and Physiology

The human nasal passage is approximately 12 cm long and runs from the nostrils to nasopharynx. The nasal cavity is divided into right and left halves by a midline septum, a cartilaginous wall. Each half of the nasal cavity consists of three well-separated regions: (i) vestibule, (ii) the olfactory region, and (iii) respiratory region. The nasal cavity has a capacity of 15 mL and a surface area of 150 cm² (31). Air generally travels through the nose in a parabolic pattern from the nose entrance to the nasopharynx. A hard palatine bone and a soft palate form the floor and roof of the nose, respectively. In humans, the nasal epithelial surface is mainly covered by stratified squamous, olfactory, and respiratory epithelium. The stratified epithelium lies in the anterior part of the nose and becomes a pseudostratified columnar epithelium at the posterior part, the respiratory epithelium. The olfactory epithelium covers the olfactory region located in the upper part of the nasal cavity. The nasal epithelial surface is covered by a continuous layer of mucus, secreted by various mucosal and submucosal glands. The mucus layer consists of two layers: sol layer and gel layer. The sol layer is a low viscosity fluid that surrounds the cilia, and the viscous gel layer covers the tips of the cilia. Cilia are fine, hairlike structures that move in an organized fashion to ease the flow of mucous across the epithelial surface. Each cilium consists of two central protein microtubules surrounded by nine microtubule pairs (31,32).

The nasal surface is supplied with a dense network of blood vessels from the external and internal carotid arteries. The blood from the anterior portion of the nose is drained through the facial vein but the main part of the nose is drained through the sphenopalatine foramen into pterygoid plexus or via the superior ophthalmic vein (32). The nasal blood vessels can be greatly dilated with blood to facilitate warming and humidification of inspired air in response to different conditions. Nasal blood flow is very sensitive to a variety of agents when applied topically and systemically. Nasal secretion contains a mixture of secretory materials from the goblet

cells, nasal glands, and lacrimal glands. The main constituents of nasal secretions are water with 2–3% mucin and 1–2% electrolytes. Nasal secretions contain several enzymes including cytochrome P-450-dependent monooxygenases, leucine aminopeptidase, lysozymes and steroid hydroxylases, and protease inhibitors (33). The normal pH of nasal secretions ranges from 5.5 to 6.5 in adults and from 5.0 to 6.7 in children. Nasal pH can vary depending on the pathological conditions including allergic rhinitis and environmental conditions such as cold and heat (34).

3.2. Factors Affecting Nasal Drug Absorption

Absorption of drugs from nasal cavity may be affected by various factors including nasal physiology, pathological condition of the nose, physico-chemical properties of drugs, dosage form, and the delivery method. While the choice of a dosage form for nasal delivery is a multifaceted issue, the presence of pathological conditions further complicates nasal drug delivery. Further, intimate contact of nasal mucosa with the atmosphere makes the nose more prone to variability in nasal absorption with the changes in temperature and humidity. Behl et al. (35) have reviewed the factors that affect nasal drug absorption.

3.2.1. Physiological Factors Affecting Nasal Drug Absorption

Nasal blood flow: The nasal blood vessels play an important role in conditioning the inhaled air. In essence, air is heated and humidified by the nasal blood flow in the direction opposite to incoming airflow. Nasal blood flow also controls the size of the nasal passage lumen. Changes in the ambient temperature and humidity, nasal administration of vasoactive drugs, nasal trauma and compression of the vasculature that supplies the head and neck may adversely affect blood flow in the nose (36). Mood fluctuation, hyperventilation, and exercise can also affect nasal blood flow and, subsequently, the nasal passage (37,38). Any change in blood flow may alter the absorption of a nasally administered drug.

Nasal enzymes: Drugs administered nasally may undergo extensive enzymatic degradation by the enzymes present in the nose (35). For example, nasal decongestant, essences, anesthetics, and nicotine are metabolized by nasal P450-dependent monooxygenase system (39,40). In addition to P450 enzymes, oxidative phase I and conjugative phase II enzymes are also present in the nasal mucosa. The phase I enzymes present in nasal mucosa include aldehyde dehydrogenase, carboxyl esterase, and carbonic anhydrases. Phase II enzymes include glucuronyl and sulfate transferase and glutathione transferase. Drugs that are known to be shown to be metabolized by the nose include progesterone, testosterone, and insulin (41,42).

Effect of mucociliary clearance: Mucociliary clearance is the movement of the mucus layer that covers the nasal epithelium toward the throat by the movement of cilia. The mucociliary clearance mechanism protects respiratory system from untoward effects of inhaled substances. An increase in the mucociliary clearance rate decreases residence or contact time between drug and the nasal epithelium, and consequently reduces drug absorption. Therefore, drug absorption can be enhanced by increasing the nasal residence time. Residence time can be increased by using bioadhesive polymers and microspheres, or by increasing the viscosity of the formulation (43). Hydroxypropyl methylcellulose, polyacrylic acid, and hyaluronan enhance absorption by increasing nasal residence time (44–46). The impact of mucociliary clearance may vary depending on the site of drug deposition. The ciliated epithelium is present in the middle and posterior region of the turbinates, but there is little or no ciliary epithelium in the anterior regions of nasal cavity (47,48). This is one of the reasons why a drug deposited in the posterior part of the nose is washed away more quickly than a drug deposited in the anterior site of the nasal cavity (49). Readers interested in the details of nasal mucociliary clearance and its role on nasal drug absorption are directed to an excellent review by Marttin et al. (43).

Pathological condition of the nose: Pathological conditions of the nose such as allergic rhinitis, polyposis, and infection may significantly influence nasal drug absorption. Most pathological conditions of the nose are frequently associated with bleeding, excessive secretion of mucus, nasal blockage, and crusting. Excessive nasal secretion may wash away the administered drug before absorption can occur (50). Similarly, the presence of nasal polyps and blockage can affect nasal drug absorption and distribution. However, several studies showed that certain nasal pathological conditions do not affect nasally administered peptide drugs. Studies with buserelin and desmopressin showed similar nasal absorption profiles in normal subjects and in those suffering from colds or rhinitis (51,52).

Dose volume and site of disposition: The human nose can accommodate a dose of 25–200 μL per nostril. Consequently, a dose exceeding this volume will be drained away. The formulator of a nasal drug delivery device must be aware of this limitation in dose volume. Nasal absorption may also be affected depending on area of the nose that is targeted by the formulation. Formulation in the anterior part of the nose provides greater contact between nose and drug, although drug delivered to the anterior portion is removed rapidly by mucociliary clearance (53). However, permeability of the posterior portion of the nose is higher than that of the anterior portion. Depending on the formulation, drugs may be administered either in the anterior or posterior part of the nose.

3.2.2. Physicochemical Factors Affecting Nasal Drug Absorption

Like any mucosal route of drug delivery, it is generally recognized that the absorption of a drug across the nasal mucosa is a function of its physicochemical properties such as molecular weight, lipophilicity, and water solubility.

Drug lipophilicity: Reports on the effects of drug lipophilicity on nasal absorption are conflicting. Nasal absorption of a series of barbituric acids has been studied in order to determine the effects of lipophilicity on nasal absorption. The study showed that absorption through nasal mucosa increases with the increase in the partition coefficient. Interestingly, there was only a four-fold increase in absorption between phenobarbital and barbituric acid despite the fact that the partition coefficient of phenobarbital was 40-fold higher than that of barbituric acid (54). Similarly, other investigators have shown that the nasal absorption of hydrocortisone, testosterone, and progesterone increases with the increase in partition coefficient. However, a hyperbolic, rather than linear relationship was observed between the *in vivo* nasal bioavailability of a series of progesterone derivatives and their octanol-water partition coefficients (55). Kimura et al. (56) obtained conflicting results when studying on the effect of lipophilicity on nasal absorption. For a series of quaternary ammonium compounds structurally related to tetraethylammonium chloride, nasal absorption was inversely related to partition coefficient. Collectively, these studies suggest that lipophilicity may not be an appropriate indicator of the extent of nasal drug absorption.

Drug solubility and dissolution: The aqueous solubility of a drug is an important factor in nasal absorption since the nasal mucosa is constantly wetted by secretions and is well perfused with blood vessels. Although the effects of solubility on oral absorption of drugs have been studied extensively, there are limited data regarding the effect of solubility on nasal absorption. Tonndorf et al. (57) compared the relative effectiveness of nasal atropine and hyoscine by measuring their capacity to arrest salivary secretion. The study showed that 0.65 mg hyoscine, a compound with 40-fold higher aqueous solubility compared to atropine, was equivalent to 2 mg atropine. Some authors suggested the presence of aqueous pores in the nasal mucosa plays a major role in nasal absorption of hydrophilic drugs (58). Dissolution is an important determinant when nasal formulations are administered as inhaled powder or suspension. Behl et al. (35) pointed out that particles from a nasal powder formulation needed to be dissolved in the nasal secretion for absorption to occur. If the drug remains undissolved in the nostril for an extended period of time, it is likely that mucociliary clearance may expel the particle out of the nose. Unfortunately, there are no

published studies that address the influence of dissolution rate of dissolution on nasal absorption.

Molecular weight: Both the size and shape of a drug molecule can affect nasal absorption. In general, molecules with an average molecular weight less than 1000 Da are better absorbed nasally than molecules with molecular weight higher than 1000 Da. Also, linear molecules have lower absorption compared with cyclic molecules (59). Several groups have investigated the effect of molecular size on nasal drug absorption (60,61). Donovan et al. (60) studied the molecular weight dependency of nasal and oral absorption of polyethylene glycol-600, -1000, and -2000. The results showed that there was a decrease in both nasal and oral absorption with an increase in the molecular weight. Likewise, studies with 13 di-iodo-L-tyrosine-labeled dextran showed an inverse relationship between the percent absorbed after nasal administration and the molecular weight of dextran. A 36-fold increase in molecular weight produced an 88-fold reduction in nasal absorption (62). The effect of molecular size on nasal absorption has been extensively reviewed by Donovan and Huang (63).

pH and pK_a : The pH of the formulation and nasal cavity, and the pK_a of the drug substance are important considerations for obtaining a reproducible absorption of drug after nasal administration. To minimize nasal irritation, the pH of nasal formulation should range between 4.5 and 6.5. However, the pK_a of the compound should be taken into account to reduce drug ionization. Further, some enzymes are capable of destroying certain bacteria at acidic pH, but at alkaline pH, they lose their bactericidal property and make the nose susceptible to bacterial infection. The effect of pH on the nasal absorption of benzoic acid was studied by Hussain et al. (54). The authors showed that the absorption of benzoic acid is pH dependent, being higher when the pH is lower than the pK_a . Conversely, absorption was reduced, the pH increased beyond the pK_a .

3.3. Models for Nasal Drug Absorption

In vivo nasal absorption has been studied in different animal models including the rat, rabbit, dog, sheep, and monkey. Readers interested in animal models for intranasal delivery are referred to an excellent review by Gizurason (64).

3.4. Nasal Drug Delivery Systems

The choice of nasal drug delivery system depends on the following: physico-chemical properties of the compound, indication, intended effect (local or systemic), the delivery devices to be used, and patient compliance. There are five basic formulations that can be used for nasal drug delivery: solution,

suspension, emulsion, gel, and dry powders. More recently, microspheres have also been proposed as nasal drug delivery system.

3.4.1. Nasal Solutions

Solutions are the most widely used dosage form for nasal administration. Nasal solutions are mainly water-based but may also contain alcohol or other solvents. Like any other liquid formulation, water-based nasal solutions are susceptible to microbial growth, so preservatives are added to prevent growth of microorganisms. The presence of preservatives may cause impairment of mucociliary clearance, and subsequent irritation. A short residence time is another disadvantage of nasal solutions. Adding viscosity-inducing materials can increase nasal residence time. Other agents that may be present in a nasal solution include buffering agents, tonicity adjusters, and solubilizers. Nasal solutions may be available as nose drops and nose sprays. While nose drops are simple to prepare and easy to use, lack of dose precision is one of the major disadvantages of this delivery system. Potent drugs that are delivered for systemic effect may not be suitable for administration as nose drops. Nasal solutions can also be delivered as sprays by using sophisticated and precise delivery devices such as metered dose pumps and actuators. Metered-dose pumps can accurately deliver a dose volume between 25 and 200 μL . Several formulation factors including viscosity, surface tension and droplet size may affect both dose accuracy and spray pattern. Nasal solutions can also be sprayed using a squeezed bottle. However, dose accuracy and site of deposition of formulation may vary depending on the pressure applied on the bottle. Nasal sprays deposit the drug more anteriorly than nasal drops, and hence the bioavailability from nasal sprays is higher than nasal drops.

3.4.2. Nasal Suspensions

Nasal suspensions are not used as widely as nasal solutions. The particle-size distribution of a nasal suspension should be carefully tailored to prevent nasal irritation. Like a nasal solution, nasal suspensions may also contain preservatives, antioxidants, and suspending agents. Nasal suspensions may provide longer residence time compared to nasal solution. Suspensions can also be delivered by using metered-dose nasal actuator system.

3.4.3. Nasal Gels

Gels are highly viscous solutions or suspensions. Although there are several advantages of a nasal gel over a nasal solution, limited work has been done with this type of nasal formulation. Behl et al. (35) pointed out several advantages of the nasal gel formulation including reduction of postnasal drip and anterior leakage. One of the major disadvantages of a nasal gel would be discomfort associated with the presence of a semisolid substance in the nose. Further, a gel is likely to block the nasal passage, thereby affecting

the normal breathing process. The absence of delivery devices that can administer an accurate amount of gel into the nasal cavity has probably hindered the development of nasal gel formulations. Vitamin B₁₂ nasal gel has recently been introduced in the US market for systemic delivery.

3.4.4. Nasal Powders

Powders are not commonly employed nasal formulations despite their advantages of being more stable and free from preservatives. However, a nasal powder formulation could potentially cause severe discomfort including irritation, a gritty feel, and dryness. Further, these formulations are difficult and expensive to produce. Compared to solutions, powders could prolong contact time in the nasal mucosa. Powders can be administered from several devices, the most common being the insufflator. Pressurized metered-dose inhalers used for pulmonary delivery can also be used for nasal powder delivery.

3.4.5. Bioadhesive Microspheres/Formulations for Nasal Delivery

Microsphere formulations have shown promise for nasal drug delivery. Bioadhesive materials that have been used to prepare microspheres for nasal delivery include dextran, starch, hyaluronan, and chitosan. Most of the compounds that have been tested for microsphere-based nasal delivery are peptides and proteins that work systemically. Starch microspheres, for example, significantly increased the nasal absorption of insulin, growth hormone, and desmopressin. Some low molecular weight drugs have also been tested for microsphere-based nasal delivery. Bioadhesive microspheres significantly increase residence time compared to nasal solutions. The application of microspheres for nasal drug delivery has recently been reviewed (65). Chitosan, a high molecular weight polysaccharide derived from chitin, has shown tremendous potential for nasal delivery of vaccines (32,66). These agents can strongly bind with the negatively charged sialic acid group of the mucin, thereby acting as mucoadhesive agents.

3.5. Absorption Promoters in Nasal Drug Delivery

One of the major challenges to nasal delivery of macromolecules is their impermeability across the nasal mucosa. Absorption enhancers have been extensively studied for their efficacy in enhancing nasal absorption of peptide drugs. Absorption promoters increase nasal absorption by increasing membrane fluidity, opening tight junctions and inhibiting proteolytic enzymes. A wide variety of absorption enhancers have been tested for nasal delivery of peptide and protein drugs. Cyclodextrins, fusidic acid derivatives, phosphatidylcholines, bile salts, and surfactants are most widely used

to enhance absorption of peptide drugs administered nasally. This aspect of nasal drug delivery has been reviewed by several authors (35,67,68).

4. PULMONARY DRUG DELIVERY

Recently, the lung has emerged as a route of choice for both local and systemic drug delivery because of its extensive surface area, thin epithelial layer, and rich blood supply. Although the use of the lung as a route of drug delivery dates back to the early civilization, its potential for systemic drug delivery of therapeutic macromolecules has been studied over the past three decades. The lung has also attracted much attention as the target organ for gene delivery to treat conditions such as cystic fibrosis and lung cancer (69). Pulmonary delivery has several advantages over oral and parenteral delivery including avoidance of degradation of the drug in the gastrointestinal tract and circumvention of the first-pass effect. Further, the pulmonary route requires a lower dose to achieve the same therapeutic response compared to other routes of drug delivery. Drug administered to the lung may produce systemic fewer side effects and can provide rapid onset of action. Pulmonary drug delivery systems are needle-free non-invasive systems, and can be administered without assistance of trained healthcare professionals. Further, pulmonary delivery has improved therapeutic acceptance and causes less disruption in the patient's life style. One of the disadvantages of pulmonary route is that sophisticated delivery devices are required to ensure accurate dosing of a drug. As a result, pulmonary drug products are relatively expensive.

4.1. Anatomy and Physiology of Respiratory Tract

Anatomically, the human respiratory tract ranges from external nares to bronchioles and alveoli. However, pulmonary drug delivery targets the tracheobronchial region and gas exchange region of the lung. The tracheobronchial region begins at the larynx and includes the trachea and the ciliated bronchial airways down to the terminal bronchioles. The pulmonary gas exchange region consists of respiratory bronchioles, alveolar ducts, alveolar sacs, atria, and alveoli. Also, the respiratory tract is divided into upper and lower respiratory regions. The upper respiratory tract consists of the nose, nasal passages, mouth, eustachian tube, pharynx, esophagus, and larynx. The trachea and bronchia are sometimes classified as part of the upper respiratory tract. The lower respiratory tract consists of the gas exchange region (i.e., bronchioles and alveoli). The alveoli are surrounded by polyhedral structures, and the side of the alveoli that is exposed to atmosphere is surrounded by thin-walled epithelium. It is important to note that trachea is divided to form smaller bronchi that lead to lung lobes. Inside each lobe, the bronchus is further subdivided to form bronchioles (70).

The major respiratory airway epithelium is pseudostratified and columnar, and the epithelium of terminal bronchioles is cuboidal in shape (71). Respiratory airways are covered with ciliated cells, and the surface of the ciliated cells is covered by cilia. Further, a thin layer, called the mucous blanket, secreted from goblet cells, covers the wall of the entire respiratory tract. The mucus is a viscoelastic tacky fluid that in conjunction with the cilia is responsible for the removal of particulates from the tracheo-bronchial region. In fact, the lung has an efficient self-cleansing mechanism, similar to the nose, called a mucociliary escalator. The mucus gel layer floats above the sol layer and the cilia extend through the mucus layer so that the tip of the cilia protrudes into the gel layer. The main component of mucus appears to be mucopolysaccharide complexed with sialic acid (33). Mucus also contains mucin, electrolytes, and water. Pulmonary mucus is slightly hyperosmotic and has a pH between 6.0 and 7.6. The alveolar epithelium and capillary endothelium have a very high permeability to water, gases, and hydrophobic substances. However, large molecular weight and ionic substances are relatively impermeable across the respiratory epithelium. The pulmonary artery supplies the lung with deoxygenated blood and the lung tissue receives oxygenated blood from the bronchial arteries. Smaller capillaries originated from the main arteries provide blood supply to the terminal bronchioles, respiratory bronchioles, and alveoli. The total surface area of alveolar capillaries is 60–80 cm² and the total capillary blood volume is 100–200 mL. The large surface area of alveolar capillary bed allows rapid absorption of large number of drug molecules from the alveoli–capillary membrane.

4.2. Factors Affecting Drug Deposition and Absorption via the Pulmonary Route

4.2.1. Particle Morphology

Drug carrier particle size of pulmonary formulations is a critical factor for the efficacy of drugs administered via the pulmonary route. Since a majority of these formulations are composed of particles of different sizes, drug absorption and distribution within the lungs is directly related to the mass median of aerodynamic diameter (MMAD) of the particles. It is worthwhile to note that MMAD is a complex parameter, which does not reflect real particle dimension. Rather, it is the equivalent diameter of a sphere moving as the real particle. In other words, MMAD includes particle properties such as size, density, and shape. The optimal particle size range for reproducible drug delivery to the lung is 1–5 μm (72). Particle size and the rate of nebulization can significantly influence the deposition pattern, and consequently affect the therapeutic efficacy and extent of systemic absorption. Little research has been done to study the influence of particle shape on drug deposition. Drug formulations with elongated drug particles are believed

to provide more selective delivery to the lung periphery (73). However, other studies suggest that particle shape has little or no influence on the respirable fraction of the drug (74).

4.2.2. Delivery Device Characteristics

The delivery device is another important factor that significantly affects drug deposition and systemic absorption from the lung. All drug formulations or drug substances may not be suitable for a particular device. Devices that are widely used for local and systemic pulmonary delivery include nebulizers and dry powder inhalers. Based on the device used, particle size, deposition patterns, and MMAD may vary, and this may eventually affect drug absorption and therapeutic effect. For example, particle size is inversely related to gas flow rate in jet nebulizers, while with ultrasonic nebulizers, the particle size is a function of the capillary waves produced on the surface of the liquid (75,76). Large variability in particle size and mass output have been observed with various nebulizers. For example, Matthys and Köhler (77) showed that mass output may vary from 47% to 81% depending on the brand and type of nebulizer. Compressor flow of the nebulizer and viscosity of inhalation solution can also affect absorption and deposition of drug administered via the pulmonary route.

Similarly, dry-powder inhalers may affect drug deposition in the lung. In powder formulations, lactose is used as bulking and deagglomerating agent. During inhalation, the inspired air aerosolizes the blend of lactose and drug, and subsequently separates the drug crystal from the lactose. Drug deposition from dry-powder inhaler may also vary depending on the type of inhaler, drug formulation, and inspiratory flow rate (78). A comparative study between Spinhaler[®] and Rotahaler[®] using a radiolabeled sodium cromoglycate/lactose blend showed that drug deposition from Spinhaler[®] was 16.4%, while from Rotahaler[®], the deposition was 6.2% (79). Zanen and Laube (78) pointed out that efficient delivery of a dry powder inhaler depended on the inspiratory flow rate generated by the patient. Increase or decrease in the flow rate may affect drug deposition by increasing or decreasing MMAD and mass output.

4.2.3. Pathophysiology of the Lung

Drug deposition in the lung may be significantly affected by the condition of the patient's airways. If the airway is significantly obstructed by mucus, there will be increased deposition at the site of obstruction, with little airflow and aerosol deposition distal to the point of obstruction (80). The deposition of particles in the central airways can be increased by only a moderate increase in the luminal mucus, causing a reduction in the penetration of aerosols through the peripheral airways (81,82). Any condition that changes the viscosity of the mucus can change the deposition of drug. It is generally believed that a reduction in mucus viscosity with mucolytic agents enhances

the penetration of aerosols into the peripheral pathways (83,84). A formulation adjuvant or pathological condition that alters the mucociliary mechanism of the respiratory apparatus can adversely affect drug deposition and absorption. Disease conditions that impair mucociliary clearance include chronic obstructive airway disease, asthma, cystic fibrosis, and bronchiectasis. Most of these conditions reduce mucociliary clearance of the lung. Absorption enhancers and preservatives are frequently found to cause mucociliary impairment.

4.3. Models for Assessing Systemic Drug Absorption After Pulmonary Delivery

Various *in vitro* and *in vivo* models for assessing pulmonary drug delivery are available for evaluating drug absorption from the lung or deposition of drug in the lung. Two inexpensive and widely used methods are the *in vivo* rat model and isolated perfused rat lung model. *In vivo* measurement of lung dose and deposition pattern can be studied by imaging and scintigraphy. Readers interested in methods for assessing *in vivo* lung dosing and deposition pattern of drugs in the lung are directed to two excellent book chapters by Clark and Borgstrom (85), and Everard and Dolovich (86).

4.4. Pulmonary Drug Delivery Systems and Devices

One of the most important requirements for inhalation drug delivery systems is that the delivery device and formulation are able to generate a respirable dose of therapeutics agent. Additional patient and formulation factors that should be considered for rational design of pulmonary drug delivery systems include physicochemical properties of the drug (e.g., pK_a and $\log P$), drug stability, interaction with the device, and the potential for aerosolization. Additionally, the patient population, clinical objective, and regulatory issues are also important. The most widely used pulmonary delivery systems are nebulizers, metereddose inhalers, and dry-powder inhalers.

5. CONCLUSIONS AND IMPLICATIONS FOR PRECLINICAL DRUG DEVELOPMENT

This chapter illustrates the profound influence of formulation and administration route on the safety and efficacy of a drug candidate. An early understanding of the pharmacological site of action and the drug exposure profile that will produce efficacy with minimal toxicity is a paramount objective.

When possible, administration at or near the site of action is ideal. For example, this may be accomplished by dermal, pulmonary, or vaginal administration. If the site of action resides within an internal organ, a route of administration that maximizes systemic bioavailability should be considered. While the oral route would be implied by this need, other routes such

as nasal or pulmonary administration may be preferable. The oral route subjects a drug to numerous barriers—chemical, physical, enzymatic—that may not otherwise be relevant with non-oral administration.

Likewise, formulation plays a very significant role in the preclinical development of a drug candidate. Liquid, semisolid and solid dosage forms nearly always contain excipients that will stabilize and sometimes modify solubility or improve the bioavailability of the active pharmaceutical ingredient (API). Yet, these excipients may have properties that go beyond improving the delivery of the API. Excipients may also possess irritation/inflammation potential. Or, they may alter permeability of barrier membranes resulting in systemic exposure to environmental or ingested toxicants.

Thus, the preclinical scientist must consider many factors when determining the most appropriate route of administration and formulation. Complexity of formulations leads to higher risk and cost during scale-up and commercialization. The route of administration must be amenable to all potential patients and provide a net convenience and accessibility that is relative to the frequency and duration of dosing, the severity of the disease, and the cost of the delivery system.

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Assessment of Pharmacokinetics and Drug Activity: Isolated Organ Systems and the Membrane Transporter Family

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1. INTRODUCTION

The relationship between pharmacokinetics and drug activity is well established. Clearance, the most important determinant of drug disposition, dictates the amount of drug that must be administered to maintain therapeutic effectiveness. Likewise, the ability of a medication to distribute to the bio-phase will influence its efficacy and/or toxicity. Each of these processes depends on drug transport across organs and tissues in the body.

Membrane transporters perform a central function in drug disposition and activity. Significant advances in experimental methodology have resulted in the identification of transporters in the liver, gastrointestinal (GI) tract, kidney, and central nervous system (CNS). Together with the metabolizing enzymes (e.g., cytochrome P-450), membrane transporters form a primary defense mechanism against the potential toxic effects of xenobiotics (1). Knowledge of the transporter(s) responsible for the elimination of a compound allows for the elucidation of potential drug interactions (drug–drug, drug–disease) and the identification of possible mechanisms of

toxicity. Furthermore, modulation of these transport systems can elicit changes in distribution, clearance, and bioavailability and, consequently, drug activity.

In this chapter, the membrane transporters that are involved in drug disposition will be presented. The impact of membrane transport on pharmacokinetics and drug activity will be discussed for individual organ systems (e.g., hepatobiliary, GI, kidney, CNS). A comparison of the experimental techniques used to study drug transport will also be provided. Finally, the implications of this topic for preclinical drug development will be summarized.

2. OVERVIEW OF MEMBRANE TRANSPORTERS

Table 1 summarizes the transporters that have been identified in the four major organ systems and will be discussed in this chapter (liver, kidney, intestine, CNS). Although Heidenhain (46) first proposed the phenomenon of drug transport in 1870, identification and characterization of membrane transporters have only evolved over the past 25 years (and continue to evolve). A general overview of each of these transport “families” is given in what follows. The role of these transporters in specific organs is described later in this chapter.

2.1. ATP-Binding Cassette Transporters

Mammalian cells possess membrane proteins that catalyze drug transport and enable cells to overcome toxicity by limiting intracellular drug concentrations. Multidrug resistance (MDR), a major challenge for cancer chemotherapy, is defined as when cells develop resistance to a broad range of structurally and functionally unrelated compounds. This resistance is generally produced upon exposure to a single substance (47). In 1976, Juliano and Ling (48) identified a plasma membrane protein that was overexpressed in colchicine-resistant tumor cells. This protein was called P-glycoprotein (Pgp). Today, Pgp is known to have a wide tissue distribution in the body (14,15,49,50).

P-glycoprotein belongs to a family of membrane transporters known as the ATP-binding cassette (ABC) superfamily (51). Comprehensive reviews of the ABC superfamily are provided in the literature (12). Two members of the ABC transporter family have been associated with MDR in human cancer cells: Pgp (MDR) and multidrug resistance-associated proteins (MRPs) (52). Two Pgp proteins have been identified in humans (MDR1 and MDR3) and three Pgps have been identified in mice (mdr1a, mdr1b, and mdr2). MDR1, mdr1a, and mdr1b are known to confer MDR (3). Although the mechanism is not completely known, these proteins presumably function as “hydrophobic vacuum cleaners,” efficiently

Table 1 Summary of Membrane Transporters Involved in Drug Disposition

Organ system	Transporters	Functional role	References
Hepatobiliary system	OCT1	Hepatic uptake of small (type I) cations	2–4
	NTCP	Bile acid transporter—a potential route for hepatic drug delivery	5–7
	OATP family (OATP 1, 2, 3)	Transporter with broad overlap (Type II cations, anions, steroids)	3, 4, 7–10
	OAT2, OAT3	Sinusoidal uptake of anions	5, 11
	Pgp	Biliary secretion of cations/MDR substrates	3, 7, 8, 12–15
	MRP2 (cMOAT)	Biliary secretion of organic anions/glucuronides	5, 12, 16, 17
	MRP1, MRP3	Sinusoidal efflux—important role during cholestasis, ↓ MRP2 expression	5, 7, 18
Gastrointestinal tract	PEPT1	Intestinal transport of di/tripeptides/ACE inhibitors/ β -lactam antibiotics	19–21
	Pgp	Intestinal efflux of xenobiotics to limit bioavailability	22–24
	BCRP1	ABC efflux transporter linked to decrease bioavailability	25
	MRP2, MRP3	Role in intestinal exsorption	18
	Miscellaneous	OATP3	6
		MCT1	21
		Anionic/cationic transport systems yet to be identified	22
Kidney	OCT1/OCT2	Basolateral uptake of organic cations	26–29

(Continued)

Table 1 Summary of Membrane Transporters Involved in Drug Disposition
(Continued)

Organ system	Transporters	Functional role	References
	OCTN1, OCTN2	Luminal transport systems	29
	Pgp	Luminal transport of organic cations/MDR substrates	28, 30, 31
	OAT1	Basolateral transport of organic anions	26, 27, 32, 33
	PEPT1/PEPT2	Luminal Reabsorption of di/tripeptides	34, 35
	OAT-K1, K2	Luminal transport of organic anions	36
	MRP2	Luminal transport of organic anions	12, 33
	OATP1	Carrier mediated reabsorption of organic anions (urine → cell)	33
	PEPT1	CNS uptake of di/tripeptides	37
	MCT family (1–8)	CNS uptake of monocarboxylic acids	37, 38
	OCT	CNS uptake of organic cations	26, 37
Central nervous system (CNS)	Pgp	CNS efflux of organic cations/MDR substrates	39–43
	OATP1	Luminal transport in BCSFB	42
	OATP2	Basolateral transport in BBB	9, 42
	MRP family (1,4–6)	Expressed in CNS	40, 41
	Miscellaneous (OAT, OCT, OCTN)	Expression shown in CNS Role of these transport families is likely to be confirmed through future studies	37
	Other transport pathways	Absorptive/receptor-mediated endocytosis of peptides Anionic/cationic efflux systems	44, 45

Table 2 Substrates for Pgp

Therapeutic class	Examples ^a
Analgesics	Morphine
Antiarrhythmics	Amiodarone, lidocaine, propranolol, quinidine
Antibacterials	Ceftriaxone, erythromycin, quinolones
Antifungals	Itraconazole, ketoconazole
Calcium channel blockers	Diltiazem, nifedipine, verapamil
Cancer chemotherapeutic agents	Actinomycin D, daunorubicin, doxorubicin, etoposide, paclitaxel, vincristine, vinblastine, tamoxifen, taxol
Fluorescent dyes	Rhodamine 123
HIV inhibitors	Indinavir, saquinavir, nelfinavir
Hormones	Dexamethasone, prednisone, progesterone
Immunosuppressant agents	Cyclosporin, FK-506
Local anesthetics	Bupivacaine
Cardiac glycosides	Digoxin
Others	Tricyclic antidepressants, surfactants, ^b grapefruit juice

^aExamples of compounds in each therapeutic category. This is not a complete list.

^bCremphor-EL.

Source: Data obtained from Refs. 13, 22, 23, 30.

extruding drugs from the cell (53,54). Although MDR3 and *mdr2* do not appear to mediate drug transport, these membrane proteins may act as phospholipid flippases and function in biliary excretion (55). However, recent data suggests that MDR3 is indeed able to transport drugs in addition to phospholipids (14). A list of known substrates for Pgp is provided in Table 2. In this chapter, Pgp refers to the transporter MDR1.

P-glycoprotein has been extensively studied in recent years. Through this research, much is known about the tissue distribution of the protein and its importance in pharmacology as a protective mechanism against potentially toxic compounds. Extensive reviews on this topic have been published (12–15). A great deal of this information was generated through studies with Pgp-knockout mouse models, mutant strains of mice that are genetically deficient in *mdr1a*, *mdr1b*, or both genes. P-glycoprotein is expressed in apical membranes of a number of organs and tissues including the intestine, liver, kidney, blood–brain barrier (BBB), testes, and placenta. The importance of Pgp as a barrier to oral drug absorption and CNS distribution has been confirmed through studies comparing drug disposition in mutant vs. “wild-type” mice. The knockout model has also helped to establish the role of Pgp in drug clearance.

As discussed later in this chapter, modulation of Pgp activity can have potentially dramatic effects on drug uptake into tissues. This is perhaps a byproduct of the intensive research efforts aimed at improving cancer chemotherapy. Indeed, inhibition of this transport system may provide the key to improving oral bioavailability and enhancing organ uptake of therapeutic moieties. A number of Pgp inhibitors have been identified. The initial Pgp inhibitors to be identified, including verapamil and cyclosporin, were relatively poor inhibitors *in vivo*. Moreover, significant side effects are observed with these therapeutically active medications, thereby restricting plasma concentrations that can be safely utilized in patients. In recent years, second and third generation Pgp inhibitors have been developed that exhibit a strong ability to inhibit the transporter without other pharmacodynamic effects. Included in this category are SDZ PSC 833 (Valspodar[®]), an analog of cyclosporin, and GF120198. The implications of Pgp modulation on drug development will be discussed at the end of this chapter.

Like Pgp, MRP transporters are expressed throughout the body (56). Multidrug resistance-associated proteins differ significantly from both in terms of amino acid sequence (only 15% overlap) and in term of substrate specificity. Whereas Pgp substrates are nonionic lipophilic compounds, MRP substrates include conjugates (glutathione, glucuronide) and other organic anions (57). Consequently, MRP proteins play a crucial role in the export of conjugated drug metabolites out of cells (12). To date, six MRP transporters have been identified (MRP1–6). These proteins are all organic-anion pumps but differ in substrate specificity and membrane location (i.e., basolateral vs. apical).

MRP1 is known to confer MDR against a wide range of cancer cells. MRP1 is expressed in the basolateral membrane and transports a wide range of substrates including glutathione-, glucuronide-, and sulfate-conjugates of medications (13).

The most widely studied MRP is MRP2, also known as the canalicular multispecific organic anion transporter (cMOAT). MRP2, an ATP-dependent transport system located on the canalicular membrane of the hepatocyte, transports glutathione, glucuronide, and sulfate conjugates into the bile (58). MRP2 is also expressed on the apical membrane kidney and intestine cells, contributing to renal secretion and gut excretion of medications (59,60). In the liver, MRP2 is believed to be involved in the biliary excretion of conjugated bilirubin. Patients with an MRP2 gene mutation (Dubin–Johnson syndrome) suffer from inherited conjugated hyperbilirubinemia.

Like MRP1, MRP3 is a basolateral transporter. MRP3 is expressed in the liver, intestine, and kidney and is believed to play a role in the movement of drugs from the cell to the blood (e.g., sinusoidal efflux in the hepatocyte). Under cholestatic conditions, MRP3 is upregulated, suggesting a role in the removal of toxic organic anions (5,13).

The most recent ABC transporter to be discovered is breast-cancer resistance protein (BCRP). Breast-cancer resistance protein was first isolated from a breast cancer cell line, although the protein is not specific to breast cancer cells. In fact, the role of BCRP in breast cancer has not been determined. It appears that, like MDR1, BCRP not only plays a significant role in drug transport, but also may be possible to modulate BCRP activity through inhibitors *in vivo* (13). As the distribution of BCRP has extensive overlap with MDR1 and MDR1 inhibitors (e.g., GF120198) also inhibit BCRP, it seems plausible that BCRP contributes to MDR in cancer cells. Further, BCRP may influence drug disposition. The importance of this protein to drug development will likely be elucidated in the near future.

2.2. Organic Anion Transporters

2.2.1. Organic Anion-Transporting Polypeptide

Organic anion-transporting polypeptides (OATPs) are a family of proteins capable of transporting medications and endogenous substances (10). To date, six OATPs have been identified in rodents (designated numerically as OATP1, OATP2, etc.) and eight OATPs have been characterized in humans (designated alphabetically as OATP-A, OATP-B, etc.). The reason for the distinction in nomenclature is that OATPs from humans and rodents are not orthologous (i.e., amino acid sequences are quite different among species), and the classification was created to avoid confusion. Species differences in transporter expression and orthology are an important issue for drug development; that is, can uptake studies (using nonhuman transporters) be extrapolated to predict drug disposition *in vivo*?

The importance of OATPs to drug disposition is still evolving and remains to be clarified. Despite the designation, OATP substrates are not only limited to organic anions, but also include cations and neutral and zwitterionic compounds. Substrates include enalapril, temocaprilat (ACE inhibitors), pravastatin (HMG-CoA reductase inhibitor), fexofenadine (H₁-antagonist), and digoxin (a cardiac glycoside).

Organic anion transporting polypeptides are found in the organs and tissues important for drug disposition and activity. While OATP-A is expressed in the brain (and in low levels in the liver), OATP-B has a broad tissue distribution in humans (liver, kidney, lung, intestine, placenta). OATP-C (also termed liver specific transporter or LST1) is predominantly expressed in the liver and is considered to contribute significantly to organic anion transport (18). On the basis of known substrates for the protein (bile acids, bilirubin), OATP-C is believed to play an important physiologic role. OATP-C is expressed in the sinusoidal membrane of the liver along with OATP-B and OATP-8. In the kidney, two transporters somewhat related to OATP have been identified: OAT-K1 and OAT-K2 (36).

2.2.2. Organic Anion Transporter

The rat organic anion transporter (rOAT1) is expressed mainly in the kidney tubule cell. rOAT1 is the dicarboxylate exchange protein that is responsible for basolateral uptake of organic anions such as *para*-aminohippurate (PAH). Substrates for this transporter include antibiotics, antiviral nucleoside analogs, and nonsteroidal anti-inflammatory medications. Over 100 medications and toxic compounds have been found to interact with rOAT1 (33).

In rats, three isoforms of OAT1 have been identified: OAT2, OAT3, and OAT4. rOAT2 is expressed primarily in the sinusoidal membrane of the liver, where it presumably functions in hepatic uptake. rOAT3 has a broader tissue distribution including the brain, liver, and kidney. Unlike OAT1, OAT2, OAT3, and OAT4 are independent of sodium and glutarate. This suggests, therefore, that these proteins function as facilitators of drug transport (along a concentration gradient) (33).

2.3. Organic Cation Transporters

Organic cation transporters (OCTs) exist in both basolateral and luminal membranes of epithelial cells. This family of proteins (OCT family) mediates the facilitated transport of cations including many medications. Six OCT proteins have been identified. rOCT1 is expressed in the basolateral membrane of the liver, kidney, and intestine (OCT1 is expressed primarily in the liver in humans). rOCT2 is also basolateral transporter that is found in the kidney and brain. Both OCT1 and OCT2 are thought to contribute significantly to drug clearance in vivo. A third basolateral transporter, OCT3, has been identified, but little is known regarding organ distribution and role in drug transport.

Three additional OCT proteins have been identified: OCTN1, OCTN2, and OCTN3. OCTN1 is an organic cation/proton exchange transporter with wide distribution in the body. OCTN2 is a sodium-dependent transporter involved in the transport of carnitine (29). The expression of these proteins in the luminal (brush border) membrane of the kidney suggests that they play a role in cation secretion.

2.4. Peptide Transporters

Another transporter superfamily of interest in terms of drug disposition is the peptide transporter superfamily. Transporters in this category include PEPT1, which plays a role in nutrient uptake and drug transport in the small intestine. In addition to di- and tripeptides, this intestinal transport system also mediates the absorption of peptidomimetic drugs (19–21). In the kidney, a high-affinity peptide transporter (PEPT2) has been identified which functions in peptide and drug reabsorption (35,61,62). Additionally,

peptide transporters in the BBB aid in the translocation of peptide molecules into the CNS.

2.5. Nucleoside Transporters

The nucleoside transporters are a group of proteins that are important for drug disposition. The kidney and intestine possess these transport proteins, responsible for the transport of nucleoside bases and nucleoside analogs (63–66). Two types of transport systems exist: equilibrative and concentrative (21). Whereas equilibrative proteins are involved in facilitated diffusion, concentrative transport proteins are energy dependent.

The equilibrative bases, including hENT1 and hENT2, possess broad substrate specificities. These proteins are expressed in the basolateral membrane of many tissues (67). The concentrative nucleoside transporters, CNT1 and CNT2, participate in sodium-dependent transport. Substrates for nucleoside transporters consist of both endogenous and synthetic nucleosides, including many medications used to treat cancer and viral infections (33). Recent data suggests that renal equilibrative and concentrative transporters mediate active reabsorption of nucleosides by the kidney (68).

3. EXPERIMENTAL METHODS USED TO STUDY MEMBRANE DRUG TRANSPORT

Recent technological advances should be credited for producing the wealth of information that has been obtained regarding the identification and characterization of membrane transporters and assessing their impact on drug disposition and activity. [Table 3](#) summarizes and compares the various experimental approaches available to study drug transport. Although the application of these methods in high-throughput screening of new chemical entities is discussed in [Chapter 10](#), a summary of experimental methods that are referenced in this chapter is provided here.

3.1. Pharmaceutical Molecular Biology

Cell-culture methodology has been applied to study numerous aspects of drug transport over the past decade. Caco-2 cells, derived from human adenocarcinoma cells of the colon, have been widely used to study intestinal transport and metabolism of xenobiotics (69). A list of available cell lines used to characterize organ transport is provided in [Table 4](#). Potential disadvantages of these isolated cell lines include diminished activity of certain enzyme systems and reduced transport activity compared with in vivo.

Gene transfection of isolated cell lines has allowed for characterization of specific membrane transporters and is particularly useful in identifying

Table 3 In Vitro Methods for Studying Drug Transport

Method	Description/applications	Comments
Cell culture	Various cell types available including Caco-2 and LLC-PK1. Gene transfection of isolated cell lines allows for characterization of specific membrane transporters and is particularly useful in identifying potential substrates for these systems	Useful for rapid identification of substrates for specific transporters Potential disadvantage is reduced transport activity compared with in vivo
Membrane vesicles	Useful method to study membrane transport from various organs (liver, kidney)	Membrane vesicle preparations are useful in identifying active transport processes involved in drug disposition
Suspended cells	Cell suspensions (liver, kidney) are freshly prepared. Can be used to probe drug-transport mechanisms	Limited value in light of alternative methods
Isolated perfused organs	Numerous applications including study of drug metabolism, elucidation of mechanism of organ transport, and assessment of drug interactions	Versatile technique that allows contribution of individual transport mechanisms to be elucidated
Mutant/transgenic animals	Animal strains lacking a specific transporter. Examples include Pgp-knockout mouse and TR- (MRP2 deficient) rats.	Comparison between “mutant” and “wild-type” animals is an excellent tool for assessing impact of specific transporters on in vivo disposition

potential substrates for these systems. Transporters that have been successfully transfected include the human intestinal peptide transporter (hPEPT1) into Chinese hamster ovary cells (72,73) and Pgp transfection of the polarized pig-kidney epithelial cell line LLC-PK1 (79–82). An overview of

Table 4 Survey of Cell Lines/Transfected Cell Lines Used To Study Drug Transport

Cell Line	Transfections	Applications	References
Caco-2 (human adenocarcinoma cells)		Intestinal transport/metabolism	69
(CHO) Chinese hamster ovary cells	CHO/hepT1	Peptide transport (PEPT1) Pgp-mediated transport Peptide transport (PEPT1)	70 71 72, 73
HepG2 (hepatic cancer cells)	MRP2	Organic anion cellular transport	74
Hepatocytes			
Collagen sandwich		Preserved bile acid uptake	75
Suspended cells		General hepatic uptake mechanisms	76–78
LLC-PK1 (porcine kidney cells)	L-MDR1/L-mdr1a	Pgp-mediated transport	79–82
	LLC-rPEPT1	Peptide transport (PEPT1) Peptide transport (PEPT2)	83 84
SKPT (renal proximal tubule cells)			
MDCK (canine kidney cells)	MDR1-MDCK	Pgp-mediated transport	57, 85–87
	cMOAT	Organic anion biliary transport	88
Xenopus laevis oocytes	rOCT1, hOCT1, hOCT2	Organic cation transport	89–91
	N1	Nucleoside transport	92
HeLa	HOCT1	Organic cation transport	93
	Npt1	Organic anion hepatic transport/efflux	94
Brain capillary endothelial cells (BCEC)		BBB transport	95
		Pgp-mediated efflux	96

available transfected cell lines for the study of drug transporters is provided in Table 4.

There are two general applications of cell culture for preclinical screening: accumulation assessment and drug-transport characterization. A review of these methods can be found in the literature (97). Accumulation studies can be used as a rapid screen for transporter activity by identifying compounds that affect the transport of a probe compound. The probe

compound is a known substrate for the transporter of interest. For example, rhodamine-123 is a useful probe for Pgp transport. This allows one to determine potential drug–drug interactions or identify compounds for possible use in cancer. By comparing drug uptake in transfected (or drug-induced) vs. “wild-type” cells, accumulation studies can also be used to identify the role of a particular transport system in drug uptake.

Transport assays study the movement of compound across a polarized epithelial barrier. These systems comprised confluent cell monolayers that are cultured on a permeable support matrix. Under these conditions, tight junctions are maintained. As in accumulation assays, drug transport is studied in transfected (or drug-induced) cells and compared to “wild-type” cells. The transport of compound is studied from both basolateral and apical direction. Bidirectional differences in drug permeability identify the presence of membrane efflux. Follow-up inhibition studies can be used to confirm these findings.

3.2. Membrane Vesicles

Another *in vitro* method for screening compounds for transporter activity is drug uptake across membrane vesicles. Membrane vesicle preparations are useful in identifying active transport processes involved in drug disposition. Vesicles, prepared by differential centrifugation, have been used to study drug transport by the intestine (98,99), liver (100–102) and kidney (61,103). In the liver, vesicles can be prepared from either basolateral (sinusoidal) or canalicular membranes. Likewise, renal drug uptake can be examined using basolateral membrane vesicles and brush-border membrane vesicles. This diversification allows for the study of various mechanisms of membrane transport.

3.3. Isolated Perfused Organs

Isolated perfused organ techniques (e.g., liver, intestine, kidney) offer a distinct advantage over other *in vitro* methods, as the intact organ is kept viable in an environment that is devoid of extraorgan influences on drug transport. Among the available *in vitro* methods to drug transport, perfused organ techniques allow for elucidation of the overall contributions of transport mechanisms on drug disposition. Therefore, perfused organ studies can provide a bridge between *in vitro* findings and *in vivo* disposition.

Isolated perfused organs have been used to study numerous aspects of drug disposition including identification of potential drug interactions. In this regard, application of perfused liver (104–108), kidney (109–114) and intestine (115–119) is well documented. Perfused rat intestine studies can be utilized as an indirect assessment of *in vivo* permeability (120). Additionally, dual

perfused intestine–liver preparations have been developed for simultaneous study of absorption and first-pass metabolism (121,122).

3.4. Whole Animal Models (Transgenic Animals and Mutant Strains)

Animal models have been extremely useful for the study of organ transport (Table 3). Specifically, genetically deficient and mutant animal strains have been successfully utilized to identify mechanisms involved in xenobiotic transport and their effect on drug disposition and activity. The absence or malfunction of a single gene defines these animal models. Perhaps, the most significant of these strains is the MDR1-knockout mouse, a model used to study the role of Pgp in drug disposition and activity (82,123–125).

Additional mutant rat strains have been established with an inherently defective ability to excrete organic anions into bile (due to MRP2 deficiency). These include transport-deficient (TR[−]) and Eisai hyperbilirubine-mic (EHBR) rats (126,127). Comparison between mutants and normal rats allows for study of the role of MRP2 on drug disposition (13,18).

4. HEPATOBILIARY TRANSPORT

Although widely recognized as the major site of drug biotransformation, one of the main functions of the liver is the formation of bile. Hepatobiliary transport processes contribute to the disposition of a number of endogenous substances and xenobiotics. Hepatic xenobiotic disposition involves a number of different pathways including uptake into the hepatocyte, intracellular translocation, biotransformation, and egress into blood and/or bile (77). Understanding the processes governing hepatic uptake and biliary secretion will not only permit a more accurate characterization of drug disposition *in vivo*, but also may allow for the development of medications specifically targeted to various regions of the liver.

4.1. Organic Cation Transport

Traditionally, organic cations were regarded as a homogenous class of compounds transported by a single transport system in the liver. Today, organic cations represent a heterogeneous group of compounds that are taken up by the liver through multiple transport systems with widely overlapping substrate specificity. Organic cations are classified into five general categories (Table 5): endogenous compounds, Type I cationic compounds, Type II cationic compounds, Pgp substrates, and cardiac glycosides. Type I compounds are relatively small, aliphatic compounds with a positively charged nitrogen group. Type II compounds are more bulky compounds with one or

Table 5 Classification of Organic Cations: Hepatobiliary Transport

Classification	Description	Examples
Type I	Small, aliphatic compounds	t-BuMA; <i>N</i> -methyl-imipramine; cimetidine; nizatadine; azidoprocaïnamide methiodide
Type II	Large, bulky cations	Vercuronium; quinidine; quinine; rocuronium
Endogenous compounds		Thiamine; choline; <i>N</i> -methylnicotinamide; d-tubocurarine
MDR substrates	Substrates for Pgp	Doxorubicin; vincristine; vinblastine; colchicine; paclitaxil
Cardiac glycosides	Unique class of medications	Digoxin; quabain

Source: Data obtained from Refs. 2–4, 50, 55, 79.

two charged groups in or near ring structures. Mechanisms of hepatic uptake and biliary egress are summarized in what follows. Detailed reviews of organic cation transport are found in the literature (3–7).

4.1.1. Sinusoidal Uptake of Organic Cations

A schematic representation of transport mechanisms for organic cations across the sinusoidal membrane is provided in Fig. 1. Sinusoidal transport of cationic compounds is bidirectional (5). The concentration gradient across the sinusoidal membrane (blood → liver) is maintained by intracellular metabolism, sequestration, and drug efflux. As intracellular concentrations increase, however, cationic transporters in the sinusoidal membrane can also act as efflux pumps.

A number of different pathways are involved in sinusoidal transport of organic cations, and not all are carrier mediated. Very lipophilic compounds can undergo passive uptake, the rate of which depends on lipid solubility and the electrochemical gradient across the sinusoidal membrane (128). Examples in this category include doxorubicin (107), rhodamine B (a fluorescent probe), and the anticholinergic agent depropine (129). Hydrophilic cations do not undergo passive uptake across the hepatocyte. The likely route for uptake of these agents is internalization through endocytosis (3) or carrier-mediated translocation into the hepatocyte.

Regarding to carrier-mediated uptake, recent evidence has identified several transporters (Fig. 1). Small cations (Type I) are transported by a proton-antiport system that is inhibited by choline and lipophilic cations. Cardiac glycosides and bile salts do not inhibit transport by this system that is designated rOCT1. rOCT1, a sodium-, chloride-, and proton-independent electrogenic rat organic cation transporter expressed in the liver, kidney,

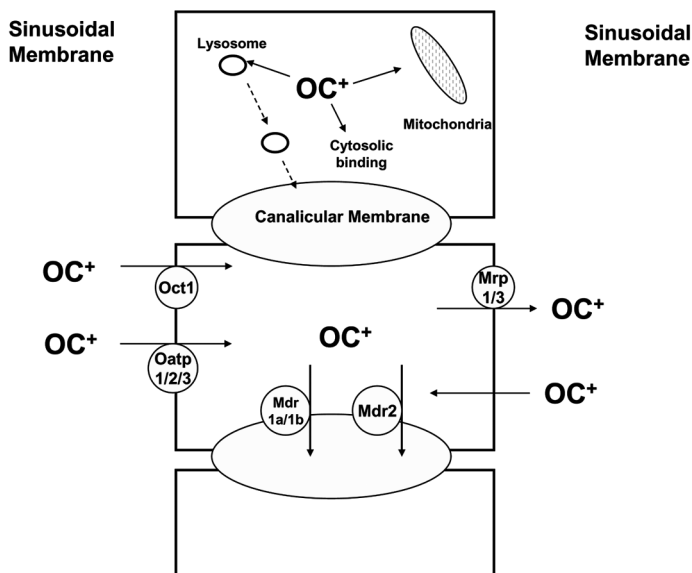


Figure 1 Schematic representation of hepatobiliary transport of organic cations. Sinusoidal transport involves passive diffusion and carrier-mediated uptake mediated by OCT1 and OATP. Once inside the hepatocyte, cations can undergo cytoplasmic binding or intracellular distribution (e.g., lysosomes, mitochondria). Biliary egress is mediated by MDR1a/1b (Pgp) and MDR2. Multidrug resistance-associated protein transporters mediated sinusoidal efflux. (Reprinted from Ref. 3, with permission from Elsevier Science.) (Modifications to the original figure were based on Refs. 5, 7, 11.)

and intestine, likely functions in the hepatic uptake of both hydrophilic and hydrophobic cations.

Conversely, transport of Type II cations is sodium independent, insensitive to choline and Type I compounds, but is inhibited by cardiac glycosides. The OATPs, rOATP1, rOATP2, and rOATP3, have been shown to transport Type II cations, including uncharged steroid compounds (130).

4.1.2. Intracellular Uptake of Organic Cations

Once taken up into the hepatocyte, considerable sequestration of organic cations by the cytoplasm and intracellular compartments can occur (106,131–133). The sites of intracellular accumulation are depicted in Fig. 1.

Lysosomal/endosomal accumulation of organic cations proceeds through two general mechanisms: passive diffusion and a proton:cation antiporter. Once internalized, the relatively acidic media of these organelles facilitates cation accumulation, the phenomenon of ion trapping.

Nevertheless, lysosomes and endosomes represent a low capacity storage site for cationic compounds. It is interesting to note, however, that decreased lysosomal uptake secondary to displacement by basic drugs has been shown to decrease the biliary clearance of several compounds. This finding suggests a role of these organelles in the intracellular drug transport to the canalicular membrane.

The mitochondria can serve as a major storage of organic cations for several reasons. First, mitochondria possess considerable intracellular volume. Second, cations can complex with DNA present in mitochondria. Third, the presence of a large negative membrane potential facilitates mitochondrial drug uptake. Several carrier-mediated processes for mitochondrial transport have been identified in addition to passive membrane potential driven uptake. Uptake is highly correlated with lipophilicity, and there is some speculation that there is a link between mitochondrial distribution and Pgp (134,135), as many Pgp substrates have been shown to accumulate in the organelle. Although mitochondria can resist penetration by hydrophilic cations, lipophilic drugs such as anticancer agents readily distribute in the mitochondria and potentially elicit toxic effects through inhibition of oxidative phosphorylation (2).

Another intracellular site of cation accumulation is the cell nucleus. The extent of nuclear uptake is a function of the relative affinity of the compound for nuclear DNA and the saturation of other organelles (e.g., lysosomes and mitochondria). Nuclear drug distribution depends on the amount of substance taken up by the liver. Therefore, diminished biliary excretion of potentially toxic compounds may increase accumulation of these agents to sites of toxicity.

4.1.3. Biliary Egress of Organic Cations

Carrier-mediated transport of organic cations has been demonstrated both in vivo (MDR1-knockout mice) and in vitro (isolated perfused liver, canalicular membrane vesicles). Although a cation:proton antiport transport system has been proposed to be involved in cation excretion (136), a functional role of Pgp on the canalicular membrane has been clearly demonstrated through these studies.

Table 6 provides a summary of studies through which Pgp-mediated biliary drug transport has been established. MDR1 expression (and possibly activity) appears to be regulated by protein kinase C. In addition to antitumor medications, a wide range of compounds that are substrates for Pgp include antihypertensive, anticholinergic, and antidepressant compounds. Despite overlapping substrate specificity, common physicochemical features of Pgp modulators are the presence of one or more aromatic ring structures, molecular weight > 400 Da, octanol:water partition coefficients greater than two, and a cationic charge at physiologic pH.

Table 6 Summary of Studies Demonstrating Pgp-Mediated Biliary Excretion of Xenobiotics

Substrate	Key findings	Reference
Vincristine	Biliary excretion inhibited by verapamil in IPL	108
Etoposide	Four to five fold decrease in hepatic and biliary clearance by Pgp-modulator Cremophor-EL in IPL	137
Doxorubicin Colchicine	Biliary clearance of both compounds decrease by Pgp-modulator SDZ PSC833 (analog of cyclosporin) in bile-duct cannulated rats	138
Doxorubicin	Pgp modulators, quinidine and GF120918, inhibited biliary excretion. Application of pharmacokinetic modeling to identify sites of interactions	107
TbuMA	Uptake of this Type I cation in canalicular membrane vesicles is active and inhibited by Pgp substrates (daunomycin, verapamil)	102
Vercuronium Azidoprocaïnamide TbuMA	Pgp-mediated uptake of both Type I and Type II cations demonstrated using cell culture	81
Type I Type II Pgp substrates	Studies in IPL demonstrating possible involvement of Pgp in biliary excretion of various compounds; may also reflect multiple transport systems at canalicular membrane	105

Recent evidence suggests that Type I and Type II cations also undergo Pgp-mediated biliary excretion (81,105). The lack of mutual inhibition among substrates that was observed in these studies suggests either major differences in affinity among substrates or the presence of multiple transport systems for organic cations on the canalicular membrane. Nevertheless, inhibitory activity correlates with lipophilicity.

4.2. Organic Anion Transport

Organic anions, like organic cations, represent a heterogeneous group of compounds differing in physicochemical characteristics. Organic acids are generally categorized as bile salts and nonbile salts. Most nonbile salt organic anions possess more than one negative charge at physiologic pH and are relatively amphiphilic. A schematic representation of the mechanisms of hepatic uptake and biliary transport of organic anions is provided

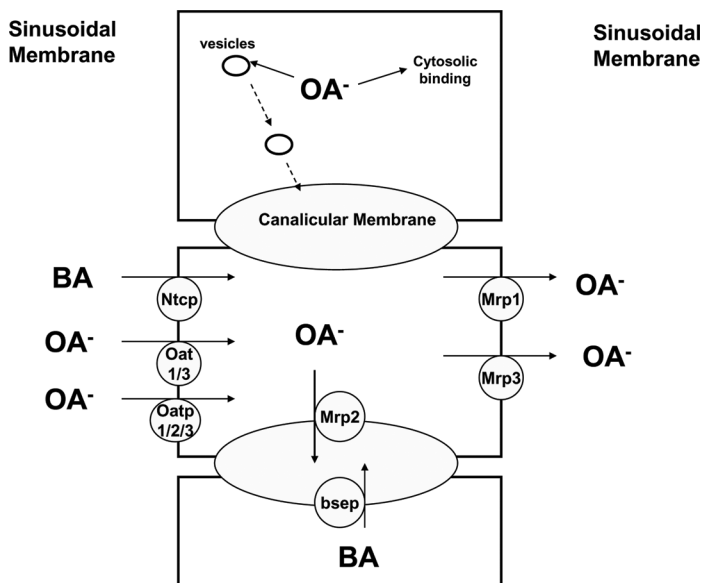


Figure 2 Schematic representation of hepatobiliary transport of organic anions. Sinusoidal transport is bidirectional. Several transport systems for basolateral uptake have been proposed: a bile acid transport system (NTCP), the multidrug transporter (OATPs), and (OATS). Once inside the hepatocyte, cations can undergo cytoplasmic binding. The role of vesicles on drug trafficking has also been proposed. Biliary excretion mechanisms include BSEP (bile acid transporter) and MRP2 (cMOAT). Other members of the MRP family (MRP1 and MRP3) are involved in sinusoidal efflux. (Reprinted from Ref. 3, with permission from Elsevier Science.) (Modifications to the original figure were based on Refs. 5, 7, 11.)

in Fig. 2. A summary of these mechanisms is provided. Excellent reviews of this topic have been published elsewhere (3,5,8,139).

4.2.1. Sinusoidal Uptake of Organic Anions

Several anionic transport systems have been identified on the sinusoidal membrane (Fig. 3). Bile-salt uptake proceeds through a sodium-dependent membrane transport system, NTCP, a widely studied system. This bile-acid transporter has not been implicated in drug transport. However, this system has been studied as a route for targeted delivery of chemotherapeutic agents to the liver by covalent binding with taurocholate (6). The effectiveness of this strategy will depend on the degree of transporter expression in cancerous hepatic cells. Additionally, recent evidence in rats has demonstrated that NTCP can be used to target nitric oxide-releasing medications to the liver, a useful treatment for cirrhosis (5).

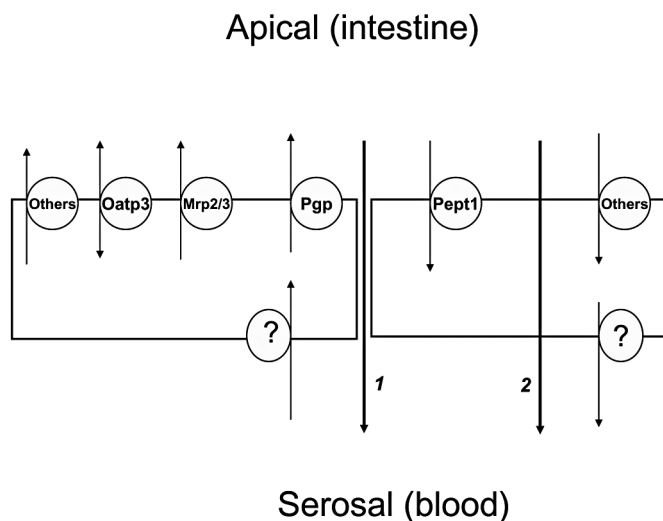


Figure 3 Schematic representation of transport mechanisms for intestinal transport. Included in this figure are both apical-to-serosal (absorption) and serosal-to-apical (exsorption) pathways. Passive absorption can proceed through paracellular (1) and transcellular routes. Di- and tripeptide transport across the apical membrane is mediated by PEPT1. Efflux transporters expressed on the apical membrane include Pgp, MRP2/3, and OATP3 (a bidirectional transporter). Other carrier-mediated transport systems that mediate intestinal transport include the nucleoside transporters (N1, N2), MCT1, and BCRP. (Reprinted from Ref. 22, with permission from Elsevier Science.)

Nonbile salt organic anions, including xenobiotics, are substrates for a sodium-independent mechanism. This broad-substrate system, designated organic anion-transport protein (OATP), participates in the transport of cations, steroids, and cardiac glycosides (see earlier). Organic anion-transport protein may be one of a family of membrane transport proteins with overlapping substrate specificities. Besides OATP, additional transport systems may be involved in organic anion uptake. Members of the organic anion transporter (OAT) family are present in liver. Although OAT1, the OAT in the kidney, is not expressed in the liver, OAT2 and OAT3 are present in the sinusoidal membrane. Substrates for OAT2 include PAH, aspirin, and methotrexate (18). Additionally, the sodium-phosphate cotransporter Npt1 has been shown to participate in the translocation of β -lactam antibiotics and probenecid (68).

The influence of extracellular albumin on hepatic uptake of organic anions has also not been clearly defined. The efficient hepatic uptake of compounds that are highly protein bound suggested a facilitative role of

albumin on liver transport. Among the theories proposed was the potential existence of an albumin receptor on the sinusoidal membrane (140–142). Recent studies suggest that OATP is a high-affinity transport system, although uptake is decreased in the absence of albumin. Interestingly, chloride increases anion transport presumably by decreasing albumin binding. Alternatively, extracellular binding to albumin has been proposed as the driving force for sinusoidal efflux (143). Hence, the differential effects of protein binding on anion uptake and efflux are not completely understood.

4.2.2. Transcellular Distribution and Transport of Organic Anions

Upon uptake across the sinusoidal membrane into the hepatocyte, organic anions are subject to complexation with several cytosolic proteins (e.g., glutathione-S-transferase and fatty acid binding protein). Hence, the cytosol represents a potential storage compartment for organic anions. Moreover, binding within the cytosol decreases sinusoidal efflux and biliary secretion. Hydrophobic anions may be subject to additional intracellular distribution but not to the same degree as organic cations.

Besides intracellular accumulation and sequestration, there is evidence to suggest that vectorial transport of organic anions to the canalicular membrane be mediated via intracellular vesicle trafficking. Reduced biliary secretion in the presence of microtubule poisons provides indirect support of this phenomenon (144). At present, this appears to be a minor pathway for anionic transport.

4.2.3. Biliary Secretion of Organic Anions

It is well established that a primary active transporter, MRP2 (cMOAT), mediates biliary secretion of organic anions. The introduction of mutant animal strains (TR[−], EHBR) in which MRP2 is hereditarily defective resulted in much of the information gathered regarding the substrate specificity of this transport system. Canalicular multispecific organic anion transporter accepts a wide variety of organic anions including many xenobiotics such as CPT-11 and methotrexate (Table 7).

The finding that biliary secretion of certain organic anions, such as pravastatin (100) and CPT-11 metabolites (101), is maintained in EHBRs suggests that multiple transport systems may exist on the canalicular membrane as depicted in Fig. 2. Further studies are needed to confirm the presence of several distinct transport systems for organic anion secretion into bile. However, the existence of numerous drug-metabolizing isozymes (cytochrome P450 classification) has prompted speculation that a superfamily of cMOAT may likewise exist in the canalicular membrane (6). A closely related protein expressed in the canalicular membrane, BSEP, is responsible for biliary excretion of bile acids (5,18).

Table 7 Substrates for MRP2 (cMOAT)

Endogenous compounds	Exogenous compounds
Bile salts	Quinolone antibiotics (grepafloxacin, lomefloxacin)
Cholate-3- <i>O</i> -glucuronide	β -Lactam antibiotics (cefodizime, ceftriaxone)
Taurolithocholate-3-sulfate	HMG CoA reductase inhibitors (pravastatin)
	ACE inhibitors (temocaprilat)
Non bile salts	Methotrexate
Conjugated bilirubin	Campotheccins (topotecan, irinotecan)
Glutathione GSH/GSSG	Bromosulphophthalein
Cysteinyl-leukotrienes	Naphthol-1-glucuronide
2,4-Dinitrophenyl-S-glutathione	Indocyanine green
	Carboxydichlorofluorescein

Source: Information obtained from Refs. 3, 4, 11, 13, 17, 57, 78, 88.

4.2.4. Sinusoidal Efflux of Organic Anions

Organic anion uptake across the sinusoidal membrane is bidirectional, suggesting that sinusoidal membrane transport systems prevent accumulation of toxic bile salts and other cholephilic compounds during conditions of defective canalicular secretion. In addition to the transport systems involved in hepatic uptake of organic anions, MRP1 and MRP3 are expressed on the basolateral membrane. It has been suggested that these transporters play a pivotal role when transport across the canalicular membrane is blocked. The expression of MRP1 is induced during liver regeneration and under conditions of experimentally induced cholestasis. The MRP3 expression is induced by medications such as rifampin. Additionally, MRP3 expression is enhanced in the cases of MRP2 deficiency due to disease (e.g., Dubin–Johnson syndrome) or genetic disruption (e.g., TR– rats).

5. GASTROINTESTINAL TRANSPORT

5.1. Traditional View of Intestinal Absorption

The most common route for drug delivery is oral administration. The traditional view of oral drug absorption is that it occurs primarily from the small intestine and proceeds via a passive transcellular process. The small intestine represents the primary site of absorption in the GI tract because of the functional specialization of the intestinal cells (creating a large surface area for absorption) combined with the prolonged intestinal transit time.

The factors known to influence the bioavailability of an orally administered medication include the following:

- *Aqueous solubility*: Dissolution in the GI fluids is necessary for passive membrane diffusion. For dissolution rate-limited drug absorption, in vitro dissolution behavior can be correlated to in vivo drug absorption.
- *Degradation in GI tract*: Examples include acid labile compounds [e.g., penicillin, omeprazole (145)] and compounds that are metabolized by digestive enzymes (e.g., peptides). Moreover, the intestinal brush-border membrane is known to contribute significantly to presystemic drug metabolism (discussed in what follows).
- *Poor membrane permeability*: Compounds with poor lipophilicity will display poor oral absorption. Historically, correlations between octanol:water partition coefficients and intestinal permeability have been attempted with divergent outcomes.

Although these general principles continue to govern the manner in which many new drug candidates are evaluated, new insights regarding the role of the intestine as a selective barrier to drug absorption have emerged. Numerous membrane transport systems are present in the intestine to facilitate the absorption of essential nutrients, systems that may also be responsible for oral absorption of certain classes of medications. Conversely, transporters in the enterocyte also serve as detoxification mechanisms in the body, which contribute to drug clearance through intestinal exsorption. By understanding the membrane transport mechanisms involved in oral drug absorption, strategies can be developed to enhance drug delivery of poorly bioavailable compounds.

5.2. Membrane Transporters Involved in GI Absorption

Figure 3 is a schematic representation of the various routes of intestinal transport. Included in Fig. 3 are both passive (paracellular and transcellular) and active transport systems. Specific membrane transport systems involved in intestinal transport are discussed in what follows.

5.2.1. Peptide Transporter (PEPT1)

As reviewed by Walter et al. (19) and Yang et al. (20), the existence of an oligopeptide transporter on the apical surface of the intestine provides an efficient route of absorption for poorly lipophilic di- and tripeptides. The transporter has broad substrate specificity. The intestinal oligopeptide transporter (PEPT1) transports β -lactam antibiotics and ACE inhibitors, medications whose bioavailability is greater than predicted on the basis of size and physico-chemical characteristics.

In general, peptide transport is electrogenic and is coupled with H^+ . Once inside the enterocyte, oligopeptides are subject to proteolytic activity. However, basolateral transport of these poorly lipophilic peptides, albeit a relatively minor mode of transport is thought also to be carrier mediated and may involve the same transport system.

The intestinal peptide transport system could be exploited to improve oral bioavailability through a prodrug approach. In theory, a dipeptide prodrug would be absorbed across the apical membrane. Once inside the cell, the active moiety would be released by proteolysis and then be transported (either by passive or active processes) across the basolateral membrane into the blood. One example of this approach is val-acyclovir (118). However, the potential of this strategy for improving oral drug delivery of poorly absorbable compounds has yet to be recognized.

5.2.2. P-glycoprotein: Role in Intestinal Efflux and Relationship with Intestinal Metabolism

The expression of MDR1 on the apical surface of the intestine suggests a role of this transporter in intestinal transport. Indeed, drug efflux by Pgp has been shown to limit oral bioavailability of compounds such as furosemide (146) and etoposide (147). Furthermore, there appears to be a co-operative function of this efflux system and intestinal metabolism in limiting drug absorption.

Intestinal phase I metabolism has been established as a contributing factor to limit bioavailability of orally administered medications (23,148,149). Approximately 70% of intestinal metabolism is mediated by CYP3A4. Interestingly, Pgp and CYP3A4 are induced by many of the same compounds. As demonstrated in Table 8, there exists broad overlap in substrate and inhibitor specificities for these two mechanisms, suggesting that Pgp and CYP3A4 act as a concerted barrier to drug absorption. This so-called drug efflux metabolism alliance is well described in the literature (24). P-glycoprotein and CYP3A4 each function to reduce systemic exposure of substances that undergo passive paracellular transport (Fig. 4). Lipophilic compounds are susceptible to intracellular metabolism or secretion by Pgp. Additionally, Pgp may also act to extrude metabolites generated from the intestinal cell. On the basis of these considerations, it appears that oral bioavailability can be enhanced through inhibition of these two detoxification pathways. In addition to MDR modulators discussed previously in this chapter, an example of a Pgp inhibitor is Cremophor EL, a commonly used excipient in oral-dosage forms (113). Grapefruit juice, a substance widely known to increase oral bioavailability through inhibition of intestinal 3A4 activity, has been shown to activate Pgp-mediated transport (86).

The Pgp-mediated secretion also contributes to intestinal drug clearance, an often-neglected route of systemic drug excretion. The Pgp-mediated

Table 8 List of Substrates for Both Pgp and CYP3A4

Pgp inhibitors	Pgp substrates	Pgp substrates and inhibitors
Amiodarone	Dexamethasone	Cortisol
Erythromycin	Etoposide	Cyclosporin
Felodipine	Rapamycin	Diltiazem
Itraconazole	Taxol	FK-506
Ketoconazole	Vinca alkaloids	Nicardipine
Lidocaine		Verapamil
Nifedipine		
Nitrendipine		
Progesterone		
Quinidine		
RU486		
Tamoxifen		
Terfenadine		
Testosterone		

Source: Reproduced from Ref. 23, with permission from Elsevier Science.

intestinal drug clearance has been demonstrated for fluoroquinolones (117,150) and may be the primary route of excretion of digoxin in patients with severe renal insufficiency (22,116).

5.2.3. Other Intestinal Transporters

Other membrane transporters may contribute to the intestinal absorption and efflux of medications. Examples of these less studied and characterized systems include the monocarboxylic acid transporter (MCT1), OATP3, MRP2/3, and BCRP.

Members of the monocarboxylic acid transporter (MCT) family are expressed in the CNS. The intestinal transport of pravastatin and salicylate is mediated by a proton-dependent transport mechanism, presumably attributed to MCT1 (21). Likewise, the presence of OATP3 on the apical membrane of the intestinal cell suggests a role of this protein in active drug absorption in the GI tract (10). Additionally, the presence of a bile acid transporter on the brush-border membrane of the enterocyte represents a potential route for improving drug delivery through the intestine (151).

Both MRP2 and MRP3 are expressed on the apical membrane of the small intestine. In studies comparing normal and mutant (EHBR) rats, a role of these transporters on intestinal exsorption has been demonstrated (18). Breast-cancer resistance protein, a member of the ABC transporter family, has been shown to mediate apically directed drug transport in the

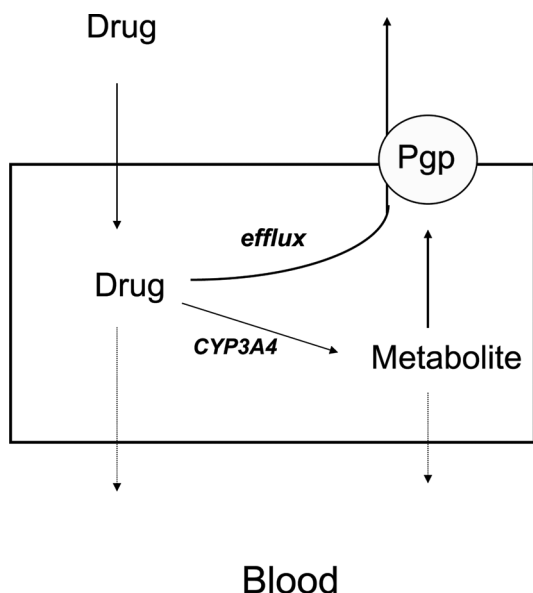


Figure 4 Model that depicts the combined role of Pgp and CYP3A4 in limiting intestinal drug absorption. Drug entering the intestinal cell via passive transcellular absorption may undergo efflux via Pgp or biotransformation via CYP3A4. Generated metabolite may also be susceptible to cellular efflux. Either metabolite or drug may be subsequently absorbed across the basolateral membrane into the blood. As intestinal content of CYP3A4 is limited, Pgp recycling may increase exposure of drug to Pgp, thereby increasing presystemic metabolism. (Reprinted from Ref. 22, with permission from Elsevier Science.)

intestine (25). Analogous to Pgp, these efflux transporters likely limit the oral bioavailability of medications.

Information is beginning to emerge about a class of transporters involved in the uphill cellular transport of nucleosides. As reviewed by Wang et al. (66), these sodium-coupled nucleoside transporters may play a critical role in absorption, disposition, and therapeutic activity of therapeutically active nucleosides [e.g., adenosine and nucleoside analogs used in to treat AIDS (e.g., 3TC and ddI) and cancer (e.g., 2CdA)]. Of the five major transporter subtypes that have been identified, two are present in the intestine (N1 and N2). The relative importance of these transporters to pharmacokinetics is unknown.

The observation that relatively polar compounds (e.g., fluoroquinolones, amoxicillin, and β blockers) appear to be substrates for Pgp and display intestinal efflux suggests the presence of transport systems on the basolateral membrane of the enterocyte. For example, the basal-to-apical

transport of celiprolol in Caco-2 cells is five times greater than apical-to-basal and is inhibited by Pgp substrates, organic cations, and organic anions (152). These transport systems remain to be elucidated but may be similar to the transport systems responsible for the secretion of xenobiotics by the kidney.

6. RENAL TRANSPORT

6.1. Overview of Renal Excretion

The kidney comprises over 1 million nephrons, the functional unit of the organ. Each nephron executes the three distinct mechanisms of renal drug excretion: filtration, secretion, and reabsorption. Approximately 25% of blood flow perfusing the kidney (450–600 mL/min) is filtered at the glomerulus. The driving force for this process is hydrostatic pressure resulting in a glomerular filtration rate of ≈ 100 mL/min. However, filtration is restrictive to only unbound, low molecular weight compounds. In addition to filtration, transport systems are present in the proximal tubule of the nephron that secretes drug into the urine across the tubular cell. The transport systems exhibit broad substrate specificity and, consequently, represent a potential site of clinically significant drug interactions. While filtration and secretion serve to excrete drug into the urine, the homeostatic function of the kidney can result in significant reabsorption of drug from the urine into the body. Tubular reabsorption is thought to be primarily a passive process, the extent of reabsorption being both urine flow rate and urine pH dependent. However, active transport systems also exist for the reabsorption of endogenous (e.g., glucose) and exogenous compounds. The transport systems involved in renal drug excretion are discussed in what follows. A number of excellent reviews of renal transport of acids and bases can be found in the literature (26–33).

6.2. Organic Cation Transport

Organic cations are transported by the proximal tubule via a multistep process, as depicted in Fig. 5. Consistent with other organ systems, the kidney efficiently secretes a wide range of positively charged medications and their metabolites. Uptake from the blood into the tubular cell proceeds by facilitated diffusion, the driving force being the electrochemical gradient across the basolateral membrane (inside negative potential difference). At least two distinct organic cation transporters have been identified on the basolateral membrane, OCT1 and OCT2 (153,154).

Once inside the tubular cell, intracellular sequestration can result in extensive drug accumulation. In addition to binding cytosolic proteins, organic cations can also accumulate in vesicular compartments (e.g., endosomes and lysosomes). The acidic pH of these organelles can

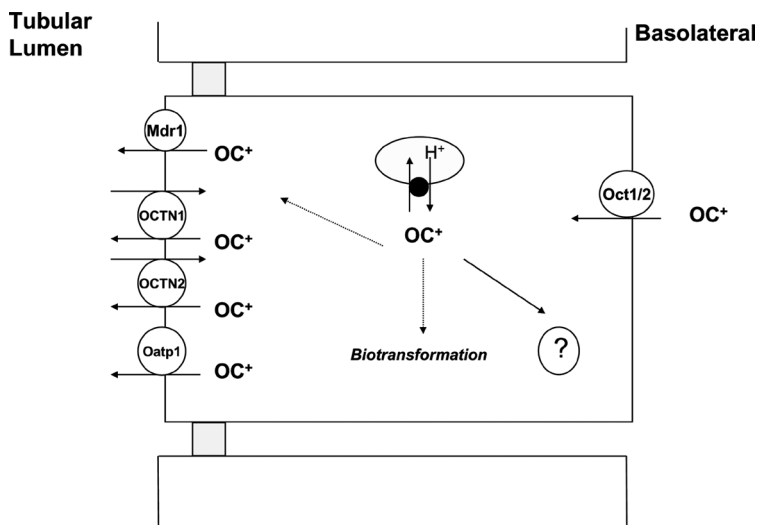


Figure 5 Schematic representation of transport of organic cations across renal proximal tubule cells. Basolateral transport is electrogenic and involves facilitated transport (inside negative potential difference) mediated by OCT1/2. Organic cations can undergo extensive intracellular sequestration. Endosomal/lysosomal transport involves both passive and active transport. The acidic pH of these organelles “ion-traps” cationic drugs. Luminal transport systems include (OCTN1/N2), Pgp, and OATP1. There appears to be considerable overlap among these membrane transport systems. Although lipophilic cations may be passively reabsorbed at more distal regions of the nephron, the possibility of carrier-mediated reabsorption exists as well (e.g., choline transport, not included in figure). (Reprinted from Ref. 27, with permission from Blackwell Science Ltd). (The figure was modified on the basis of Ref. 29.)

“ion-trap” cationic compounds that enter via passive diffusion. However, endosomal uptake of tetraethylammonium was found not only to be saturable, but also inhibited by other cationic compounds including MDR substrates (155,156). This suggests the existence of an active transport system for vesicular accumulation. The significance of intracellular sequestration on drug transport has not been completely defined, although there is speculation that vectorial vesicular trafficking may contribute to the secretory process.

Once inside the kidney cell, drug metabolism can also occur. Although the liver is the responsible for drug biotransformation, the kidney contains many drug-metabolizing enzymes and contributes to renal metabolism of a number of medications (157,158).

Luminal transport of cationic compounds into the urine is mediated by several distinct transport mechanisms. Although the transport protein

Table 9 Substrates for Renal Tubular Transport Systems: Organic Cation Transporters and Pgp

Organic cation transporter(s)	P-glycoprotein
Choline	Antracyclines
H ₂ -antagonists (cimetidine, ranitidine)	Colchicine
Histamine	Cyclosporins
Morphine	Digoxin
Nucleoside analogs (lamivudine zidovudine)	Ethidium bromide
<i>N</i> -Methylnicotinamide	Quinidine
Paraquat	Quinine
Procaine	Taxol
Quinine	Verapamil
Tetraethylammonium	Vinca alkaloids
Trimethoprim	
Verapamil	

Source: Reprinted from Ref. 28, with permission from Elsevier Science.

remains to be identified, the classic mode for cationic luminal transport involves proton exchange via a tertiary active transport mechanism (29,89,90). A Na⁺-H⁺ exchanger generates the proton gradient, with intracellular Na⁺ levels maintained through Na⁺-K⁺-ATPase. A list of substrates for this transport system is provided in Table 9.

The finding that the kidney contains relatively high levels of MDR1a Pgp combined with *in vitro* and *in vivo* studies with MDR substrates supports a role of MDR1a in the renal secretion of xenobiotics. Given the lipophilic nature of organic cations, passive tubular reabsorption of these compounds would be expected. Thus, it appears that MDR1a Pgp functions to limit reabsorption and kidney accumulation of potentially toxic medications and environmental toxins (30).

There seems to be a significant degree of overlap between these luminal transport systems (Table 9), suggesting that certain organic cations may be substrates for both systems. However, the pH dependence of daunomycin transport mechanisms demonstrated *in vitro* suggests that alterations in blood pH may dictate which transport system will predominantly contribute to drug secretion.

Several other transport systems have been identified in luminal membrane of the kidney: OATP1, OCTN1, and OCTN2. OCTN1 is an organic cation/proton exchange transporter. OCTN2 is a sodium-dependent transporter that couples cation secretion to carnitine reabsorption (159).

In addition to the earlier-mentioned transport systems mediating cation secretion into the urine, certain compounds such as choline undergo

active tubular reabsorption. Choline reabsorption is mediated by two mechanisms of varying affinities. The low-affinity transport system exhibits broad specificity and, therefore, may be an important pathway for cationic disposition. Therefore, in contrast to passive reabsorption (urine pH and flow dependent), active reabsorption of cations may be dose dependent, drug clearance increasing with increasing dose.

6.3. Organic Anion Transport

The proximal tubule of the kidney contains organic anion transport systems that secrete a wide array of exogenous compounds including many drugs (Table 10). In contrast to organic cations, tubular transport of organic anions proceeds against an electrochemical gradient at the basolateral membrane, with facilitated transport across the luminal membrane into the urine (down an electrochemical gradient). As illustrated in Fig. 6, the weak acid transport system (OAT1) is a tertiary active system. Organic anion basolateral uptake involves counter transport with α -ketoglutarate (α -KG). Outflow of α -KG occurs along two pathways: a sodium dicarboxylate transporter and intracellular metabolism.

While OAT1 is the principle anion basolateral transporter in the kidney, other members of the OAT family may also be involved, including OAT2 and OAT3. Additionally, efflux of organic anions across the basolateral membrane has been proposed. This efflux system has been linked to members of the MRP transport family (MRP 3, 5, 6).

Table 10 Substrates for Organic Anion Renal Tubular Transporters

Therapeutic class	Examples
Benzoates	
Carbonic anhydrase inhibitors	Acetazolamide, methazolamide
Cephalosporins	Cephalexin, ceftriaxone
Diuretics	Chlorothiazide, furosemide
HIV inhibitors	Zidovudine, adefovir, cedofivir
NSAIDS	Salicylic acid, indomethacin flurbiprofen
Penicillins	Penicillin G
Prostaglandins	
Sulfonamides	Sulfisoxazole
Miscellaneous	Probenecid
	<i>para</i> -Aminohippuric acid
	Methotrexate
	Bile acids
	Saccharin

Source: Information obtained from Refs. 29, 32, 33.

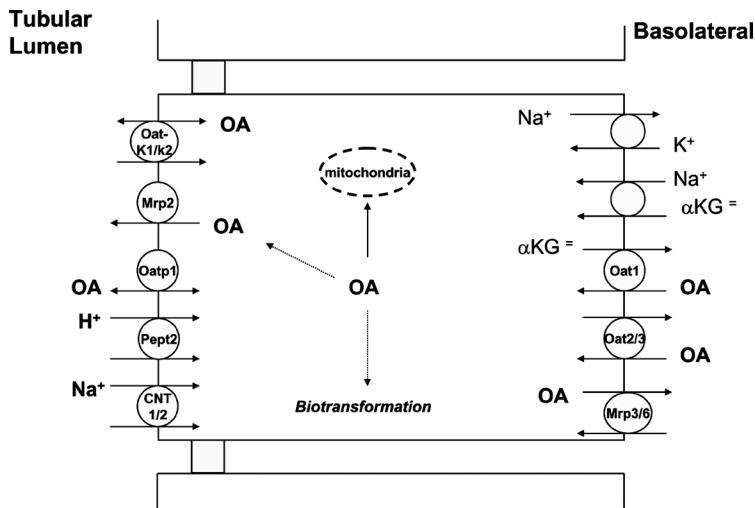


Figure 6 Schematic representation of transport of organic anions across renal proximal tubule cells. Basolateral transport is carrier-mediated. This organic anion transport system is a tertiary active system that involves counter transport with α -ketoglutarate (α -KG). Once inside the tubular cell, there is a possibility of cytoplasmic binding or distribution into intracellular compartments. Luminal transport of organic anions is electrogenic and involves facilitated transport. Several transporters have been identified including OAT-K1/K2, MRP2, and OATP1 (bidirectional transporter). Also included in the figure are transporters that mediate the active reabsorption of peptides (PEPT2) and nucleosides (CNT1/2). (Reprinted from Ref. 27, with permission from Blackwell Science Ltd). (The figure was modified on the basis of Refs. 29, 33.)

Following tubular uptake, intracellular accumulation of anionic compounds can occur (33). In addition, vesicular trafficking and cytoplasmic binding may influence anionic transport through the cytoplasm from the basolateral to luminal membrane. For example, the extensive renal accumulation of methazolamide in the isolated perfused rat kidney model can be attributed to drug complexation with cytoplasmic carbonic anhydrase (160). As stated earlier, biotransformation (phase I/II reactions) within the kidney cell is also possible. The role of intracellular distribution and metabolism on the renal disposition of organic anions requires further study.

Information is emerging regarding the translocation of organic anions across the luminal membrane into the urine (Fig. 6). Transport proceeds through a facilitated mechanism down an electrochemical gradient. A number of transport pathways have been proposed for the luminal exit of acidic compounds, although the relative contribution of these mechanisms is species dependent. Both OATP1 and OATP3 are expressed in the brush-border

membrane of the kidney. Additionally, OAT-K1 and OAT-K2 are kidney-specific transporters structurally similar to OATP1. These transporters are sodium- and ATP-independent. Although it is assumed that these are efflux transport systems (lumen $\rightarrow$ urine), they may also be involved in luminal reabsorption.

Many compounds that are secreted by the organic anion secretory system are poorly lipophilic and therefore are not significantly reabsorbed by the kidney. However, there are weak acids (e.g., salicylates) that are sufficiently lipophilic to undergo tubular reabsorption. For these compounds, urine pH and urine flow rate will be expected to influence renal excretion.

6.4. Other Renal Transporters

6.4.1. Peptide Transporters

The kidney contains the peptide transporters, designated PEPT1 and PEPT2. In contrast to the low-affinity, high-capacity, intestinal oligopeptide transporter (PEPT1), PEPT2 is a high-affinity, low-capacity transporter. However, both transport systems are similar in terms of substrate specificity. Renal disposition of β -lactam antibiotics and ACE inhibitors involves PEPT2-mediated transport (61).

6.4.2. Nucleoside Transporters

The identification of nucleoside receptors (CNT1, CNT2) in the kidney suggests a role of these systems in the renal tubular transport of nucleosides and nucleoside analogs. However, the importance of this transport system as a clearance pathway for xenobiotics remains to be determined.

7. CENTRAL NERVOUS SYSTEM TRANSPORT

Unlike the other organ systems discussed in this chapter that function in drug clearance and bioavailability, the CNS is an important region for drug targeting. However, the movement of compounds from the systemic circulation into the CNS is limited by two general barriers: the BBB and the blood–cerebrospinal fluid barrier (BCSFB). The BBB, composed of brain capillary endothelial cells (BCECs), has traditionally been viewed as a lipoidal barrier with tight junctions between cells, rendering the BBB restrictive to CNS translocation of xenobiotics. The BCSFB consists of a single continuous layer of epithelial cells that line the endothelial cells of the choroids plexus (CNS1). The tight junctions of these epithelial cells form the BCSFB barrier.

Given the nature of the BBB and BCSFB, drug transport to the brain is a transcellular process, with lipophilicity being a primary determinant of the process. For a number of compounds, however, lipophilicity does not correlate with CNS penetration; that is, drug uptake across the BBB is lower than expected (40). On the basis of recent evidence, this discrepancy is

explained by the presence of transport systems that may function in CNS uptake and efflux of xenobiotics. Understanding the key features of these pathways may allow for improved treatment of diseases of the CNS (e.g., brain tumors, bacterial and viral infections) through enhanced uptake of neuropharmaceuticals. Furthermore, CNS-related side effects of medications could be avoided by blocking these mechanisms.

The systems responsible for CNS uptake and efflux of xenobiotics are summarized in what follows. Detailed reviews are provided in the literature (37–44).

7.1. Transport Systems for CNS Uptake

Figure 7 provides a schematic representation of the various transport pathways for drug uptake into the CNS. The net uptake of drug by the CNS is determined by the difference between the rate of drug influx and the rate of drug efflux (38). Among the factors that influence drug uptake into the CNS are the pharmacokinetic properties of the drug and the ability of the compound to penetrate the BBB. As the driving force for passive diffusion is a concentration gradient, binding of drug to plasma proteins or blood cells will modulate CNS penetration. Additionally, the presence of metabolizing enzymes in the BCECs is a limiting factor for drug penetration. In addition to transcellular penetration, nondiffusible molecules can penetrate the BBB via carrier-mediated transport or endocytosis.

7.1.1. Transport of Organic Anions

The MCTs are expressed in a number of tissues, including the brain (CNS6). A total of eight MCTs have been identified to date (38). Monocarboxylic acid transporters are involved in bidirectional membrane transport across the BBB. MCT1 has been identified on the basolateral membrane of BCECs. MCT1 transports lactic acid and other short chain acids. The pH dependence of this acidic system suggests a proton-cotransport mechanism is involved.

Medications bearing a carboxylic acid moiety appear to be substrates for MCT1. One such class of medications is HMG-CoA reductase inhibitors (e.g., lovastatin and simvastatin). The CNS distribution of the less lipophilic drug pravastatin suggests a lower affinity for the transporter, resulting in a reduced incidence of CNS side effects. Overall, an understanding of the role of MCTs in the BBB is still evolving.

Another family of transporters implicated in CNS uptake is OATP. OATP1 is expressed in the luminal membrane of the BCFSB, whereas OATP2 is expressed in the basolateral membrane of the BCFSB and the brush-border membrane of the BBB. Transport across OATP2 is bidirectional.

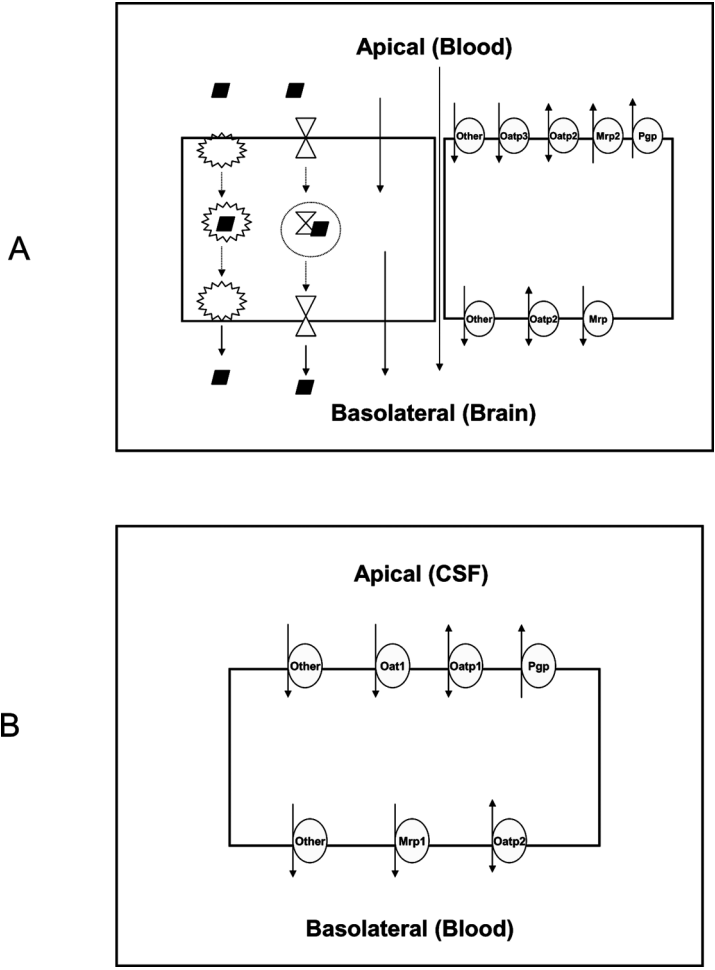


Figure 7 Schematic representation of transport mechanisms for drug (■) transport across the BBB (A) and BCSFB (B). Depicted in panel (A) are both uptake and efflux transport pathways at the BBB. (1) Absorptive endocytosis, a low-affinity, high-capacity system. (2) Receptor-mediated endocytosis, a pathway for CNS uptake of peptides. (3) Paracellular diffusion through gap junctions. This system can be modified (osmotic agents) to enhance drug transport. Among the membrane transport systems identified in the BBB are Pgp, MDR, and OATP. Transport systems have identified at the BCSFB (panel B). Transport systems at the apical membrane include Pgp, OATP1, (directional) and OAT1. MRP1 and OATP2 (bidirectional) are basolateral transport systems. Additional transport systems have been suggested at both BBB and BCSFB. (Reprinted from Ref. 37, with permission from Elsevier Science. The figure was modified on the basis of Refs, 18, 40, 41.)

7.1.2. Transport of Organic Cations

In addition to passive transport (a function of lipophilicity and molecular weight), at least two distinct carrier-mediated transport mechanisms have been identified for CNS transport of organic cations (Table 10). Analogous to MCT1, structural differences among a class of compounds, such as the H₁-antagonists, alters the affinity for these systems and, consequently, the side effect profile of individual medications.

7.1.3. Transport of Peptides

Several distinct transport mechanisms are available for the translocation of peptides (both natural and synthetic) into the CNS. Given that peptides are generally unstable, hydrophilic molecules, passive diffusion of these compounds is limited. However, the transport systems that have been characterized offer potential routes of targeted delivery of biologically active peptides to the brain. These systems are listed in Table 11.

The first type of transport mechanism is carrier-mediated transport that may be similar to the transport system that is present in the intestine and kidney (PEPT1). Although not extensively studied, as many as five different transporters may be involved. Examples of peptides that appear to be substrates for carrier-mediated uptake include enkephalin (161), vasopressin (162), and interleukin-1 (163).

Positively charged peptides can gain access to the CNS through absorptive endocytosis. An electrostatic interaction between the peptide and the negatively charged membrane generates translocation. Thus, this pathway is considered a low-affinity, high-capacity mechanism.

An additional mode of peptide transport is receptor-mediated endocytosis, in which the peptide molecule is shuttled across the BBB into brain interstitial fluid by specific receptors. Among the receptors that have been identified are an insulin receptor (164) and a transferrin receptor (165).

Both absorptive-mediated and receptor-mediated endocytosis represent promising routes for peptide delivery. For example, complexation of neuroactive peptide with cationized albumin could facilitate translocation into the CNS through absorptive endocytosis (166). Alternatively, monoclonal antibodies to a specific receptor could be used to enhance CNS uptake via chimerization (167,168). Covalent attachment of a poorly permeable compound to a suitable vector could enhance brain uptake.

7.2. Transport Mechanisms for CNS Efflux

As stated earlier, lipophilicity remains an important determinant of drug permeation through the BBB. However, there are a number of compounds that display poor CNS distribution despite high lipophilicity. Although plasma protein binding and molecular size of these compounds are possible explanations for these findings, recent evidence (e.g., transgenic animal

Table 11 Summary of Transport Mechanisms for Uptake and Efflux Across BBB

Mechanism	Description	Substrates
Passive transport	Depends on lipophilicity, molecular weight	Organic cations
MCT1	Monocarboxylic acid transporter	HMG-CoA
Organic cation transporters	Carrier-mediated uptake of cations. May involve more than one system	Reductase inhibitors β -Lactam antibiotics Quinolone antibiotics H ₁ -antagonists
Carrier-mediated peptide transport	Similar to PEPT1/PEPT2	H ₂ -antagonists Enkephalin Interleukin-1
Absorptive endocytosis	Low-affinity, high-capacity mechanism for positively charged peptides	Cationized albumin
Receptor-mediated endocytosis	Alternative mode of peptide transport across BBB	Insulin
P-glycoprotein	Broad substrate efflux system in CNS	Enkephalin
Organic anion efflux system	OATP, MRP transporters	Protease inhibitors MDR substrates <i>para</i> -Aminohippuric acid

Source: Information compiled from Refs. 34, 37, 39.

studies) points to the presence of efflux mechanisms in the BBB and BCSFB that restrict access of these compounds to the CNS. The transporters expressed in the BBB and BCSFB are depicted in [Fig. 7](#). Most notable among these transport mechanisms is *mdr1* (Pgp).

7.2.1. P-glycoprotein-Mediated Efflux

The identification of Pgp on the apical surface of BCECs suggests a role of this transport system in limiting access to the CNS. P-glycoprotein is also expressed in the apical side of the BCSFB. Indeed, a number of studies both *in vitro* and *in vivo* have supported the general concept that Pgp pumps xenobiotics from the brain interstitial fluid into the blood. [Table 12](#) provides a sampling of the compounds for which Pgp efflux has been demonstrated. Key features of the Pgp transport system such as substrate characteristics

Table 12 List of Compounds Transported at BBB via Pgp and other Transport Mechanisms

P-glycoprotein	Other transport mechanisms
Amprenavir	Baclofen
Cyclosporin A	Carnitine
Dexamethasone	Cimetidine
Digoxin	Cyproheptadine
Domperidone	Gabapentin
Doxorubicin	L-Dopa
Met-enkephalin	Lovastatin
Erythromycin	α -Methyldopa
Indinavir	Pravastatin
Ivermectin	Simvastatin
Loperamide	Taurine
Nelfinavir	Valproic acid
Ondansetron	
Pacitaxel	
Saquinavir	
Vinblastine	

Source: Obtained from Refs. 37, 39, 44.

are discussed in other parts of this chapter. As demonstrated in Table 12, the list of Pgp substrates is both chemically and pharmacologically diverse.

Much of the information regarding Pgp-mediated CNS efflux has been generated using transgenic mice lacking Pgp. The various Pgp-“knockout” mouse models are mutant strains of mice that are genetically deficient in MDR1a, MDR1b, or both genes. Knockout mice do not display physiologic abnormalities and have life span comparable to normal mice. However, CNS distribution of a number of compounds is increased dramatically in knockout mice. This is perhaps best illustrated by the pesticide ivermectin. Although ivermectin has a good safety profile in normal mice, it causes lethal neurotoxicity in knockout strains (38). Studies with knockout mice have demonstrated a role of Pgp in limiting the CNS penetration of medications used to treat HIV (e.g., indinavir, ritonavir), cancer (e.g., doxorubicin, vincristine), cardiovascular disorders (e.g., digoxin, quinidine), and pain (e.g., morphine).

7.2.2. Other Efflux Systems

In addition to Pgp, it is likely that other efflux transport systems exist in the CNS. MRP1, which confers multidrug resistance to anticancer medications (e.g., doxorubicin, vinblastine), is expressed in the basolateral membrane of the BCSFB. Expression of MRP1 in the BBB is controversial, although

studies in cultured BCECs suggests that several members of the MRP family (MRP1,4,5,6) are found in the BBB.

Recent evidence has also demonstrated expression of OAT, OCT, and OCTN transport families in the CNS. Established substrates for these transporters including acyclovir (OAT1), methotrexate (OAT1), quinidine (OCT2) are known to be actively transported out of the brain (38). Therefore, it is likely that these transport families play a role in CNS efflux. Further characterization of these transporters and their function is eminent.

8. IMPACT OF MEMBRANE TRANSPORTERS ON OTHER ORGANS AND TISSUES

A major focus of this chapter was the role of membrane transport on organs involved in absorption, metabolism, and excretion of medications. Not unexpectedly, the expression and characterization of membrane transporters in other tissues of the body are actively being pursued. For example, understanding the role of efflux transporters in the placenta will allow for the development of treatment strategies for pregnant women that minimize the risk of harm to the fetus. Likewise, understanding the role of mammary gland transporters on drug excretion into breast milk is important from a clinical and toxicological perspective. The impact of membrane transport on placenta and mammary drug transfer has been the subject of recent review articles (169,170). The impact of membrane transporters on these tissues, as well as others (e.g., testes, eyes), will undoubtedly be realized in the near future.

9. IMPLICATIONS FOR PRECLINICAL DRUG DEVELOPMENT

The objective of this chapter was to provide an overview of the various transport systems that contribute to drug disposition *in vivo*. In this context, a number of transport systems such as the bile-acid transporters were not emphasized, although they may prove to be useful routes for drug delivery. As membrane transport is a rapidly evolving area of study, detailed characterization of these and other transporters will be achieved in the near future.

Assessment of membrane transport and the role of transporters on pharmacokinetics and drug activity have significantly impacted preclinical drug development and will continue to affect the drug development paradigm in the future. Specific implications of membrane transport for preclinical drug development are provided in what follows.

9.1. Emerging Areas of Importance

The identification and characterization of membrane transporters involved in drug disposition and activity has only begun. However, there are several specific issues that will certainly impact the drug development process. These issues are noted in what follows.

9.1.1. Species Differences in Transporter Structure and Activity

Humans and rodent membrane transporters are not orthologous; that is, amino acid sequences of transport proteins are different among species. For this reason, the nomenclature of membrane transporters, such as OATP, is different between animals and man. Species differences in transporter expression and orthology are an important issue for preclinical drug development. Future research will undoubtedly determine differences in transporter activity (and substrates) among species, and cell culture preparations that express specific human transporters are available. Extrapolation of whole animal studies to predict clinical outcomes will depend on the extent of overlap in transporter expression and activity between species.

9.1.2. Pharmacogenomics of Membrane Transporters

Pharmacogenomics is the study of the relationship between genetic variations and individual differences in drug response (171). The impact of pharmacogenetics on drug metabolism is illustrated by the established polymorphisms of the cytochrome P-450 enzyme family (e.g., CYP2D6) and *N*-acetyltransferase. Likewise, polymorphism in membrane transport proteins is expected. Although there is limited information on the pharmacogenomics of drug transporters in general, the effect of polymorphism on Pgp expression and function is the subject of considerable interest.

The pharmacogenetics of MDR1 and its impact on pharmacokinetics and pharmacodynamics has the subject of recent reviews in the literature (172,173). A single nucleotide polymorphism, C3435T, has been associated with a twofold increase in duodenal expression of Pgp, resulting in reduced systemic exposure to orally administered digoxin (174,175). C3435T polymorphism has also been associated with antiretroviral treatment in HIV-infected patients, as some HIV medications (e.g., protease inhibitors) are Pgp substrates. A subgroup of patients with reduced Pgp expression (TT genotype) showed a more favorable response to treatment, presumably due to increase drug penetration into infected cells (176). MDR1 polymorphisms have been reported to be a risk factor for diseases such as inflammatory bowel disease, Parkinson's disease, and renal carcinoma (177).

Information concerning the pharmacogenetics of membrane transporters is fundamental to drug therapy, that is, achieving adequate drug concentrations at the target site with the goal of maximizing therapeutic

effectiveness while minimizing toxicity (178). In the not so distant future, drug selection and dosing will be predicated on the genetic profile of the patient. Certainly, understanding the pharmacogenomics of membrane transporters will impact pharmaceutical care in the coming years. In terms of preclinical drug development, extracting the clinically relevant information from available pharmacogenetic data will be a major challenge.

9.1.3. Gender Differences in Membrane Transport

Gender effects on drug disposition are another emerging issue for drug development. The influence of gender on pharmacokinetics and drug activity is well established, and it is likely that sex-related differences in membrane transporter expression and activity are an underlying cause of these differences. Gender differences in drug transport have been determined in organs including the liver, intestine, kidney, and CNS (179).

9.2. High-Throughput Screening for Transporter Affinity

Advances in experimental methodology (e.g., cell culture) allow for rapid identification membrane transporters that may influence in vivo disposition and activity of new chemical entity. Current high-throughput screening strategies in the areas of membrane permeability and drug metabolism are greatly enhanced with this information. Identification of specific transport systems for which an investigational compound has demonstrated affinity will enable scientists to predict potential drug interactions in vivo. Furthermore, potential pitfalls with a new chemical entity can be identified early on in drug discovery. For example, a compound with affinity for Pgp may suffer from poor oral bioavailability.

A useful algorithmic approach to identify and evaluate the impact of specific transporters on drug disposition (and activity) would involve rapid assessment of drug affinity for specific membrane transporters. In cases where specific transport pathways have been identified, follow-up studies could be conducted to confirm the significance of the transport system or to identify potential drug interactions. These studies could be conducted in vitro at the cellular, whole organ level (perfused organ studies) or in vivo using transgenic or mutant strains. In terms of the drug discovery process, information gleaned from these studies could help identify potential problems and promote rational decision-making during the drug development process.

9.3. Targeted Drug Delivery

As our understanding of membrane transport continues to evolve, innovative strategies in the area of targeted drug delivery will emerge. In the liver for example, new insights into the process of hepatobiliary excretion mechanisms could result in new approaches to capture medications in

hepatocytes or deliver them to the biliary tree. Identification of transporters in the intestine will provide critical information for formulation scientists to exploit these routes and improve bioavailability of biological products. Treatment of diseases of the kidney (e.g., renal carcinoma and polycystic kidney disease) will likewise be improved through the rational design of delivery systems based on knowledge of renal transport mechanisms. Moreover, CNS delivery of medications can be improved or CNS-related side effects of medications can be avoided through selective modulation of uptake and efflux transport systems at the BBB.

9.4. Clinical Implications of Membrane Transporters

Transport proteins play a fundamental role in drug distribution to various regions of the body. In the CNS, efflux transporters act to restrict drug access to the brain. The efficiency of these transporters dictates drug therapy in the treatment of conditions: AIDS-related dementia, epilepsy, pain management, and brain tumors (38). Transporter expression is influenced by factors such as genetics (171), gender (179), age (180), and concomitant drug therapy (181), resulting in interpatient differences in therapeutic response and toxicity.

Modulation of drug transport is a promising strategy to improve drug delivery to target areas. In the CNS, for example, inhibition of efflux transport would increase drug accumulation. Given the considerable overlap in substrate profiles of these transport proteins, specific inhibitors for individual transport systems must be developed (38).

Much of the research focus in the area of membrane transport (and also of this chapter) concerns Pgp. This is, perhaps, a byproduct of the intensive research efforts aimed at improving cancer chemotherapy. Indeed, inhibition of this transport system may provide the key to improving oral bioavailability and enhancing organ uptake of therapeutic moieties.

There have been a number of clinical studies conducted to determine whether modulation of Pgp activity improves the efficacy of cancer chemotherapy (182). Studies have shown that Pgp modulators such as verapamil and cyclosporin indeed reverse resistance in a small number of patients, but significant side effects are observed with these therapeutically active medications. Additionally, it is likely that the principal effect of these modulators may be through altered pharmacokinetics and not MDR reversal in cancer cells (183).

Presently, a number of Pgp-specific modulators are in various stages of development that demonstrate inhibitory activity but are devoid of pharmacologic effects. Examples include Valspodar (PSC833), Biricodar (VX-710), and VX-853 (38). Not only might these compounds prove useful in the treatment of cancer, but they may also be able to enhance oral absorption or CNS distribution of drug products. There is, however, a potential danger

associated with this strategy. Given that Pgp (and other transport system) is ubiquitously expressed throughout the body and serves primarily as a detoxification mechanism, is it prudent to inhibit this system? By enhancing CNS delivery through reduction Pgp-mediated efflux across the BBB, will potentially toxic compounds also accumulate? More, importantly, will this open the door for toxins ingested in the diet to gain access to the systemic circulation due to nonselective inhibition of intestinal Pgp? Alternatively, Pgp may be just one of many systems in place and can therefore be modified without risk. These issues remain to be resolved. In the meantime, the quest for the magic bullet continues.

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Alternate Methods for Assessing Absorption, Metabolism, and Routes of Elimination

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1. INTRODUCTION

This chapter will focus on the remarkable changes that have occurred during the past 10 years in our ability to evaluate many compounds simultaneously with respect to their pharmaceutical (drug-like) properties, including intestinal absorption, first-pass metabolism, and elimination. The majority of these advancements have applications at the interface between discovery and pre-clinical development, during which accurate and timely information can improve the quality of development candidates and reduce the risk of subsequent, costly failures in pre-clinical and clinical testing. Many of the alternate methods discussed herein are based on methods used for many years during pre-clinical testing. In the past, lead compounds were selected for development on the basis of pharmacological potency and efficacy in animal models, because a certain level of potency is pre-requisite to selecting a lead compound or series and, as potency increases, more delivery routes become reasonable options. Unfortunately, selecting for high potency often selects out favorable delivery properties (e.g., solubility and permeability). Historically, this strategy has failed to reliably identify potent ligands that were also well tolerated, efficacious medicines. During the last two decades, there has been a marked increase in the demand for new drugs exhibiting

improved efficacy and selectivity. An increase in the number of pharmacological targets is expected to occur as one result of sequencing the human genome. There are also expectations that new drugs can be developed more rapidly and inexpensively. These expectations have exerted immense pressure to accelerate drug discovery and development. In response to these demands, a strategy employing automated, parallel organic synthesis has been widely adopted to prepare new libraries of medicinal compounds. This was followed by the modification of existing *in vitro* binding and inhibition assays to screen these large collections (up to 10^6 members per library) for appropriate biological activity and potency against a single target (e.g., receptor, enzyme, second messenger). This primary high-throughput screen (HTS) is used to identify new high-affinity ligands, so-called hits, from among hundreds of thousands of new chemical entities (NCE). This strategy frequently identifies hundreds or thousands of biologically active compounds requiring further investigation.

New hits are also subjected to further testing against secondary targets (e.g., target sub-types, related targets) to eliminate the most promiscuous compounds—those more likely to manifest toxicity. This screen for specificity has been termed high-throughput profiling (HTP) and may be used to identify compounds that are most selective in their pharmacological effect. However, information accrued from HTS and HTP is frequently insufficient to estimate important pharmacokinetic variables (e.g., plasma half-life, bioavailability) and thereby select the NCE most likely to become marketable medicines.

The majority of medicines developed in North America are intended for oral administration. Therefore, a tertiary screening method, pharmaceutical profiling (PP), has been developed to help identify promising medicines from among large collections of selective hits and to optimize the bioavailability and minimize potential for toxic effects. These assessments are conducted during drug discovery and they frequently run in parallel with HTP evaluations, in order to further minimize the time required before full characterization. The information generated by such pharmaceutical profiling experiments has been used to rank selective hits according to physicochemical properties, intestinal absorption, hepatic metabolism, and elimination. The results of PP have the potential to minimize the losses of attrition due to the failure of lead compounds in pre-clinical studies or human trials, reduce the time to market and thereby control the overall cost of drug development. It is possible that selecting lead compounds in this way will result in the approval of efficacious and potent medications that tend to have fewer adverse side effects. This chapter describes new applications and formats of *in vitro* methods employed in high-throughput assessments of pharmaceutical properties.

2. THE EVOLUTION OF DRUG DISCOVERY AND ITS EFFECT ON PRECLINICAL DEVELOPMENT

The process of pharmaceutical innovation is complex and measures of research and development output are important in understanding trends in drug development (1). The recent history of pharmaceutical industry performance suggests that, despite the investment of billions of dollars in excellent human resources and state-of-the-art instrumentation, the development of new medicines is a risky business, one that has experienced many failures for each success. Several indices of success have been proposed including the number of NCE synthesized and the number of patents issued. However, time-to-market and the number of NCE approved in a given period of time may be the most practical measures of pharmaceutical success.

Between 1960 and 1980, development time of NCE from synthesis to market almost quadrupled and has remained relatively unchanged since 1980, with a present interval of 9–13 years (2). This has been accompanied by a corresponding decrease in the effective patent life, from nearly 13 years to 6.5 years (1). Typically, from the day of the discovery of new targets and new lead structures until the decision to proceed with full-scale development, 5–6 years have passed (3). It has been estimated that in order to discover such lead structures, an average of 50,000–100,000 NCE must be screened.

The failure of lead compounds in phase I and phase II clinical trials is a major source of scientific and economic difficulty. In Britain, over a 17-year period from 1964 to 1980, a total of 197 NCE were evaluated in man for the first time (1). However, 137 were withdrawn from development and 35 (18%) continued through development; the ratio of drugs innovated to those marketed was 5.6:1 (1). This is similar to the 5.6:1 ratio reported for the United States or 13.5:1 for Swiss companies (1). The major reasons for withdrawal included inappropriate pharmacokinetics in human subjects (39.4%) and a lack of clinical efficacy (29.3%). The incidence of unexpected toxicity or more subtle adverse effects was the third most common reason for termination (10%).

Every excess year of drug development is an unnecessary use of resources that could be applied elsewhere, the money being spent as well as the revenues being lost due to late marketing. Thus, time also becomes a most important factor in drug development (3). A rough calculation of daily revenues lost on a new medicine with average market potential suggests that each day of delay in getting NCE to market is approximately US\$2M. The increasing cost of efficient drug development is associated with prolonged development time and increasing regulatory requirements. In 1987 expenses for the development of NCE averaged US\$300M–US\$400M. Currently, in the United States, the costs of drug research and development have increased to between US\$800M and US\$1B per approved medicine (4).

As indicated above, typically a large number of NCE enter pre-clinical development for every one emerging as a new medicine (5). Furthermore, most of the escalating costs of drug development are associated with latter-stage development. Therefore, poor candidates must be identified as early as possible and immediately terminated from development. In many cases, a company's success depends on how the attrition of these compounds is managed. In order to manage attrition properly, however, the reasons why compound development slows down must be included. The drain of valuable resources by slowdowns can be much more damaging to overall success than that arising from outright terminations. Some of the slowdown factors are strategic in nature, others are strictly attributes of the individual NCE and some are attributable to how an organization makes these decisions.

Poor pharmaceutical properties contribute significantly to both failures and slowdowns in development. In fact, poor drug-like characteristics can lead to consumption of large human and dollar resources throughout the development timeline. In the past, NCE with excellent potency and selectivity, but with poor pharmaceutical properties, was rarely disqualified from entering the development pipeline. It has been estimated that a proficient medicinal chemist is capable of synthesizing 200–300 NCE per year (2). Given the historical information above, it is perhaps not surprising that screening a very large number of NCE appears to be one logical strategy to improve the output of new medicines. There is historical precedent for this approach.

The strategy of synthesis and screening of rationally designed NCE was pioneered shortly after World War I. Typical molecular scaffolds were systematically combined with many other groups that occur repeatedly among biologically active compounds. A battery of receptor binding systems, utilizing tissue homogenates, radiolabeled ligands and animal models, helped in the characterization and optimization of biological activity. More than 50 new drugs, among them analgesics, anti-histamines, neuroleptics, and anti-depressants, resulted from this discovery strategy (3). However, in the past, many drug discovery programs were based on the random screening of large series of synthetic intermediates, by-products or analogs in animal models. Mass screening in drug research essentially started with the testing of thousands of different sulfonamides for their antibacterial activities. However, from about 1970 large screening programs became less and less important (3), because the yield of new leads from random screening was considered to be too low, compared to the necessary effort. Between 1970 and 1990, the structures of potential drug candidates were more rationally planned and the synthesized compounds were commonly tested in just one or two selected models before lead selection occurred.

The integration of automated laboratory systems and modified experimental protocols, and the advent of improved data management tools and

rapid and sensitive analytical instruments, have permitted the combinatorial synthesis of a substantial number of structurally distinct compounds using similar reaction conditions (6). The process is based on systematic molecular design either by linking separate building blocks or by adding substituents to a core structure. Many large international drug companies have established extensive molecular libraries. The most positive consequence of using combinatorial methods to synthesize NCE libraries is that several drug candidates can then be developed in parallel, in order to avoid the failure of a whole program if a single compound gives a negative result in its first application to humans. Combinatorial chemistry and the enormous capacity of high-throughput screening systems allow the synthesis and testing of thousands of compounds or mixtures per week. It has been estimated that combinatorial chemistry techniques have contributed to reducing the time required to discover drug candidates by 18–24 months. However, the obvious disadvantage to this approach is that the number of drug candidates entering the pipeline may soon overwhelm development resources. Although it is sometimes claimed that combinatorial libraries are valuable also for lead structure optimization, this claim needs to be questioned because of the lack of appropriate starting materials for their synthesis. In general, there continue to be questions as to whether this combinatorial approach will actually deliver new and better medicines more rapidly than in the past (7). The balance of this chapter contains a discussion of the major elements included in the pharmaceutical profiling of NCE and how each element contributes to the selection of lead medicinal compounds.

3. LEAD SELECTION AND OPTIMIZATION VIA PHARMACEUTICAL PROFILING

Pharmaceutical profiling (PP) has become a bridge between medicinal chemistry/pharmacology and pre-clinical studies. Most of the experimental procedures are conducted in vitro in order to maximize their capacity and minimize costs. This constraint has forced the modification of existing techniques and the development of new models and techniques, including the miniaturization of cell-based and cell-free assays.

3.1. Solution Properties

The behavior of NCE in biological solutions can markedly influence their success as orally administered medicines. The early determination of physicochemical (PC) behavior (aqueous solubility, partition coefficient, and serum protein binding) provides useful information concerning absorption, metabolism, and elimination; that is, their pharmacokinetic behavior. More importantly, these criteria may be useful in identifying compounds in drug

discovery that could be potentially difficult and expensive to take through the entire development process.

With the broad implementation of combinatorial synthesis, the sources have changed significantly. Previously, lead sources included natural products, clinical observations of side effects, presentations at scientific meetings, published reports in scientific journals and collaborations with external investigators. From a PC viewpoint, the most poorly behaved compounds were typically eliminated first, leaving a lead that possessed solution properties consistent with previous experience in discovering orally active compounds. However, this process has changed significantly since 1989, with the implementation of high-throughput screening (8). This development was supported by rapid improvements in the expression of human and animal receptor sub-types and in signal detection.

3.1.1. Aqueous Solubility

There is actually a paucity of published reports on the determination of aqueous solubility of NCE during drug discovery. However, information on the aqueous solubility of NCE can be quite valuable in optimizing and selecting lead series or compounds. Low aqueous solubility is frequently associated with poor intestinal permeability, since permeation is proportional to concentration gradient. Formulation may also be difficult and time consuming for relatively insoluble NCE because the aqueous solubility of a drug influences its dissolution rate and the rate and extent of absorption. The determination of aqueous solubility normally precedes the other assessments in PP, as solubility information can determine the method used to deliver NCE to PP assays. Poor solubility alone rarely terminates compound progression, although it does result in slower development and a lower probability of success.

It appears that two factors affect the PC profile of hits arising from library preparation and HTS. First, combinatorial methods of synthesizing NCE tend to yield larger, more hydrophobic NCE and typical HTS assays are biased towards selecting less soluble compounds (7). The latter artifact arises, in part, from the use of DMSO as a universal water-miscible solvent in drug discovery, because the focus in discovery is on keeping NCE in solution rather than measuring the actual aqueous solubility. This method alters the kinetics of solubilization and the result is that NCE added to aqueous medium in DMSO is transferred in a very high-energy state. Therefore, this "apparent" solubility in HTS assays is almost always higher than the thermodynamic solubility measured by equilibration of a well-characterized solid in aqueous medium. The net result of these changes is that *in vitro* activity is reliably detected in NCE with relatively poor actual aqueous solubility. Therefore, it is possible that lower-potency hits with more favorable pharmaceutical characteristics may be discarded. Solubility effects can be further complicated by the fact that products from combinatorial organic synthesis in discovery may differ substantially in their physical form (generally much

more crude) than the purified, soluble salts available in pre-clinical development. Typically, solution spectra, HPLC purity data, and mass spectral analysis are sufficient to support the synthesis of compounds for HTS and HTP. In most cases, NCE submitted for PP are screened for aqueous solubility because the final concentration of NCE employed in a typical panel of PP assays, as described in this chapter, varies considerably (1–50 μM).

The determination of aqueous solubility in PP is commonly conducted by one of two methods, both of which may be considered relatively “higher-throughput.” One method is via turbidimetric measurement, a technique frequently labeled a kinetic solubility measurement, which ignores traditional pharmaceutical concepts of thermodynamic solubility. As currently practiced (7), a concentrated stock solution (e.g., 10 $\mu\text{g}/\mu\text{L}$ or 20–30 mM) in DMSO is added drop-wise to a small volume of isotonic, buffered saline (e.g., 2.5 mL) while the absorbance at 600–820 nm is monitored or the degree of light scattering is measured. An extended time-course is used in order to avoid missing precipitation that would affect a biochemical experiment. Precipitation is identified with a diode array UV detector by an increase in absorbance due to light scattering by particulate matter. The method is, however, quite sensitive to the juxtaposition of the cuvette and detector, and intensely colored NCE can cause false positive results. However, it does not depend on the presence of a chromophore in the molecule. In order to maintain reasonable throughput in this assay (40–50 samples per day), the precipitation point is simply calculated from a curve fit to the absorbance vs. volume of stock solution and the NCE is concentration expressed as micrograms of NCE per milliliter of buffer. The functional range of this assay is 5–65 $\mu\text{g}/\text{mL}$ and the final concentration of DMSO remains below 0.7%. Typical for assays included in PP assessments, a set of reference compounds, normally existing medicinal compounds, is screened with each assay. For example, among approximately 350 medicines selected from the Derwent World Drug Index, 87% exhibited turbidimetric solubility greater than 65 $\mu\text{g}/\text{mL}$ and only 7% had solubility of 20 $\mu\text{g}/\text{mL}$ or less. One interpretation of these results is that if turbidimetric solubility is less than 20 $\mu\text{g}/\text{mL}$, the probability of useful oral activity is quite low, unless the compound is very potent or unusually permeable, or unless it is a substrate for a biological transporter. Furthermore, if aqueous solubility is greater than 65 $\mu\text{g}/\text{mL}$, poor pharmacological activity in vivo is probably not related to solubility (7). [Table 1](#) provides examples of the kinetic solubility of several existing active pharmaceutical ingredients, as measured in our kinetic solubility (KS) assay. Currently, 40 compounds are evaluated in duplicate in a 96-well plate by adding 1.5 μL of a concentrated DMSO solution to 300 μL of potassium phosphate buffer, pH 7.4, at room temperature and the light scattering is measured with a nephelometer. The NCE are tested at 10 and 50 μM and the compounds are ranked as unacceptable (KS < 10 μM), marginal (KS = 10–50 μM) or acceptable KS (> 50 μM).

Table 1 Measurement of the Kinetic Solubility of Marketed Medicines

Solubility category	Compounds
Acceptable ($KS > 50 \mu M$)	Verapamil Metoprolol Naproxen Dapsone Clozapine Amitriptyline
Marginal ($KS = 10\text{--}50 \mu M$)	Terfenadine Estradiol Haloperidol Nicardipine
Unacceptable ($KS < 10 \mu M$)	Ellipticine Reserpine Astemizole Amiodarone Paclitaxel Miconazole

An alternate method of determining aqueous solubility and purity has been developed. It incorporates aspects of a typical thermodynamic solubilization procedure and of the turbidimetric procedure discussed above. The assay is conducted in 96-well plates, requires only a very small amount of starting material and it is capable of evaluating 80 NCE per plate. A very small aliquot of a concentrated NCE stock solution (10 mM in DMSO) is added to a small volume of phosphate-buffered saline. This solution is shaken overnight, after which the plate is centrifuged to separate the phases and an aliquot of the supernatant is injected for analysis by high-performance liquid chromatography (HPLC). A set of reference medicines, spanning the solubility range of this technique (1–100 μM), are also prepared in the same way and included in each plate. A quality control sample of each reference compound, prepared in methanol/water, is also analyzed. The throughput of this assay is supported primarily by rapid chromatography. The standard method is limited to evaluating compounds that contain a chromophore, but NCE that do not absorb UV may be analyzed by HPLC/mass spectrometry (LC-MS), as long as the compound will accept a charge. The information can be used to determine which NCE are soluble enough to be accurately assessed in other PP assays.

3.1.2. Lipophilicity

One of the most reliable methods employed by medicinal chemists to improve pharmacological activity *in vitro* is to incorporate properly

positioned lipophilic groups to change the hydrophobic interactions between a target and its ligand. Early consideration of lipophilicity and its influence on activity, transport and distribution in vivo may avoid many frustrations for chemists and biologists who optimize their drugs only based on in vitro results. An assessment of lipid solubility is frequently included in an evaluation of PC properties (9) because lipophilicity has been associated closely with biological effect in structure–activity analyses (10). Furthermore, lipophilicity appears to be proportional to molecular weight and higher-molecular weight NCE and high lipophilicity has been associated with poor intestinal permeability (11).

For nearly 50 years, the standard technique used to determine relative lipophilicity has been the measurement of octanol/water distribution coefficient using the widely accepted shake-flask method and calculation of the logarithm of the partition coefficient ($\text{Log } P$ or $\text{Log } D$). This has been used as a surrogate for the membrane partition coefficient (k_m). The value of $\text{Log } P$ may be estimated by chromatographic partitioning to determine a corrected retention volume (V_{cr}) with modified HPLC methods or the solute capacity factor (k_{IAM}) on immobilized-artificial-membrane (IAM) columns. Liposome partitioning of NCE may also be used as a surrogate for k_m . Although measurement of lipophilicity is preferred, attempts have been made to increase the throughput of lipophilicity determinations by predicting, rather than measuring, lipophilicity. More than 40 different approaches have been developed to predict lipophilicity by calculating the contribution of the molecular characteristics of compounds. The added value of octanol/water partitioning information for new NCE lies, in large part, in the continuity it provides with historical determinations of lipophilicity of successful medicines.

Originally, lipophilicity was determined in drug discovery on a small scale by determining the octanol/water partition coefficient ($\text{Log } P$) or the apparent octanol/buffer partition coefficient ($\text{Log } D_{7.4}$) experimentally with the well-established shake-flask procedure (12). Octanol, with its polar head and flexible, non-polar tail, has hydrogen-bonding characteristics similar to those of membrane phospholipids. The octanol/water partition coefficient is also thought to model the hydrophobic interactions between xenobiotics and biological membranes. Briefly, solute is added to a separation funnel, followed by equal volumes of water-saturated octanol and octanol-saturated water, the funnel is vortexed for 30 sec and then gently agitated at room temperature. After 2 hr, the phases are separated by centrifugation, the funnel is allowed to stand for 2 hr for complete separation of phases. An aliquot of each phase is analyzed for the compound of interest. However, this method is laborious and time consuming as it is currently practiced and there are serious difficulties associated with the chromatography of NCE dissolved in octanol. In addition, the measurement of partition coefficient in this way may have only limited reproducibility. This assay has been modified for

higher-throughput assessments of lipophilicity. It is conducted in 96-well plates according to historical protocols. Unfortunately, at this time, analytical challenges remain to be addressed.

Attempts have been made to improve the reproducibility and throughput of octanol/water partition coefficient measurements. Despite problems with standard HPLC measurement of partition coefficient, it has been suggested that HPLC retention data may prove to correlate with biological activity as well as, or better than octanol/water partition coefficient (13). The potential usefulness of this technique was demonstrated on a group of xenobiotics ($n=52$), among which the Log P values, as measured by shake-flask, ranged from +0.28 to +5.01 (14). The results of this study suggested that the value for Log P , estimated via a column-corrected chromatographic method, correlated very well with Log P measured via the shake-flask method ($r=0.999$). However, for maximal accuracy and minimal variability, numerous elution-time measurements are required, which reduces its applicability to high-throughput pharmaceutical profiling.

Partitioning of NCE into liposomes has been used to accurately approximate lipophilicity by measuring the membrane partition coefficient k_m . Liposome partitioning can model both polar and non-polar interactions between solute and membrane (15). However, liposome-partitioning experiments are also labor intensive, requiring the preparation of liposomes, adequate solute equilibration time, measurement of free solute in the presence of liposomes and corrections for the amount of solute that partitions into the aqueous phase. Therefore, this technique is very unsuitable for high-throughput evaluations. However, the approach appears to be a valid one, because it has been shown that liposome partition coefficient correlates well with partition coefficient in endogenous membranes (16).

Lipophilicity still represents one of the most informative PC parameters available to drug discovery. Due to the implementation of combinatorial synthesis and high-throughput biological screening, the motivation to develop new models to estimate lipophilicity rapidly was increased significantly after 1991. Immobilized artificial membrane (IAM) chromatography utilizes a solid phase membrane model, in which phospholipids (e.g., phosphatidylcholine) are covalently bound to solid support at monolayer densities. This technology had been used to purify membrane proteins, immobilize enzymes and characterize enzyme-ligand interactions. Recently, these columns have been used to measure a solute capacity factor, k_{IAM} , as a surrogate for membrane partition coefficient to obtain hydrophobicity information on existing medicines and NCE (17,18). The technique combines the advantages of using membrane phospholipids with the high-throughput advantage of chromatographic partitioning. It has been found that k_{IAM} correlates well with k_m ($r=0.907$) irrespective of Log P values or structural differences. However, this relationship does not exist between log k_{IAM} or log k_m and Log P ($r=0.419$ and 0.483 , respectively). It appears

that when non-polar interactions between solutes and membranes dominate membrane interactions, both $\log k_{\text{IAM}}$ and $\log k_{\text{m}}$ correlate well with $\text{Log } P$ (16). Conversely, when hydrophobic interactions dominate octanol/water, partition methods give the same results as IAM methods ($r=0.985$). These qualifications suggest that this technique may be applied most reliably to a series of structurally similar NCE (19) and that it should probably not be used to screen structurally diverse compounds for relative lipophilicity.

The pressure to screen several hundred NCE per day subsequently yielded an excellent example of a high-throughput discovery assay via modification of conventional IAM chromatography technique, described above (20). The major objective of creating this technique was to model human intestinal absorption, but it yielded an index of lipophilicity that was comparable to $\text{Log } P$.

IAM chromatography was a significant development for drug discovery, permitting rapid assessment of interactions between NCE and the phospholipid component of biological membranes. However, the number of hits arising from combinatorial synthesis, HTS and HTP continues to increase. At the same time, the number of new targets being identified by molecular techniques is rising. Therefore, a great deal of effort has been expended in finding algorithms to predict $\text{Log } P$. This topic was recently reviewed comprehensively (21), so the discussion of $\text{Log } P$ predictions here will remain general. Existing methods of predicting $\text{Log } P$ fall into roughly three categories. Group contribution methods (e.g., $C \log P$) dissect molecules into pre-defined fragments and their corresponding contributions are summed to obtain a calculated $\text{Log } P$ value. This method incorporates a number of correction factors, because a molecule is not just the sum of its fragments. Similarly, atomic contribution methods (e.g., $M \text{Log } P$) of predicting $\text{Log } P$ do so by considering the individual contribution of single atoms. Like group contribution methods, this reductionist approach is based on the contributions of pre-defined fragments, determined by multiple regressions on a database of experimental $\text{Log } P$ values. Molecular methods of predicting $\text{Log } P$ (e.g., $B \text{Log } P$) incorporate a description of each molecule as a whole, not just as simple fragments put together and consider the interaction between solute and solvents (octanol and water). These methods use quantum and geometric descriptors (e.g., surface area or volume) or conformational analysis. All three types of predictive methods require complex calculations. As a matter of practical application to drug discovery, current opinion (7) suggests that $M \text{Log } P$ is the most useful method for tracking the physicochemical properties of a combinatorial library of NCE, because it covers a larger variety of molecular structures with acceptable accuracy. On the other hand, $C \log P$ is most applicable to characterizing analog series, because it emphasizes accuracy of predictions and it is applicable to less diverse collections of compounds.

3.1.3. Plasma Protein Binding

The behavior of NCE in biological solutions (e.g., plasma) influences the activity, metabolism, and elimination of many medications. One important aspect of this behavior is serum protein binding, which is a pharmaceutical property of medicinal compounds. In the past, precise but experimentally laborious methods of measuring serum protein binding were developed to evaluate total binding in serum. These include equilibrium dialysis and ultrafiltration techniques to measure binding dissociation constants (K_d), and they still play a valuable role in drug development (22,23). However, chromatographic and electrophoretic methods to measure serum albumin binding can be employed as rapid screening tools for investigating drug binding in drug discovery (24). Table 2 illustrates the differences we have observed between the values for plasma protein binding in the case of several existing medications and how these compare to values reported by the original innovator company. NCE are evaluated in duplicate in 96-well plates at a final concentration of 10 μ M. This assessment is typically conducted immediately before the nomination of a development candidate.

Affinity chromatography generally uses retention time on a serum albumin stationary phase as the parameter that correlates with the degree of protein binding. These methods can be extended to the analysis of enantiomers (25,26). This procedure has been modified such that serum albumin is added to the mobile phase buffer (27). In this case, a shift to a shorter retention time in the presence of protein indicates a binding interaction of the drug and albumin. The extent of binding is measured by comparison of the shift in retention time in the presence of albumin to the retention time of the drug in mobile phase minus albumin. When compared to the results obtained by ultrafiltration, this method yields thermodynamically valid binding measurements.

Table 2 Plasma Protein Binding of Existing Medications: A Limited Comparison of Species and Methods

Compound	In vitro plasma protein binding (%)				Reported human plasma protein binding (%) ^a
	Equilibrium dialysis		Ultrafiltration		
	Human	Rat	Human	Rat	
Warfarin	99.4 ± 0.4	99.7 ± 0.2	98.9 ± 0.2	99.5 ± 0.1	99 ± 1
Verapamil	90.5 ± 2.1	93.2 ± 0.5	84.8 ± 0.5	80.0 ± 2.2	90 ± 2
Propranolol	88.1 ± 0.5	91.0 ± 1.1	86.2 ± 2.1	78.3 ± 3.0	87 ± 6
Naltrexone	61.7 ± 3.1	73.9 ± 0.7	66.1 ± 3.1	48.9 ± 2.1	20

Values are mean $\pm$ SEM.
^aValues were taken from the Physician's Desk Reference.

Affinity capillary electrophoresis, combined with a variety of detection methods, has also been used to screen drug–albumin interactions. Several versions of this basic technique have been developed (28). Frontal and vacancy peak analysis use UV detection to measure unbound drug, and the Hummel-Dryer technique uses UV absorbance to measure bound drug. In affinity capillary electrophoresis, binding parameters are calculated from the change in electrophoretic mobility of the drug upon binding.

3.2. Intestinal Permeability and Absorption

One of the major influences on the success of orally administered medicines as therapeutic products is the rate and extent of their intestinal absorption. This complex process is a function of the physicochemical properties of the drug and the permeability of the intestinal barrier that determines drug absorption. Transcellular movement of solutes occurs via passive diffusion through the enterocyte membrane and is the most significant absorptive mechanism for the majority of drugs. Membrane permeability typically depends on three interdependent PC properties, including lipophilicity, hydrogen-bonding potential, and molecular size (29). Paracellular movement of solutes also occurs via passive diffusion through the enterocyte membrane pores and at cellular tight junctions. It has much less significance in intestinal uptake of most drugs compared to the transcellular pathway.

3.2.1. Physicochemical Properties

Currently, there is a great deal of interest in evaluating the role of physicochemical properties in intestinal membrane permeation. Membrane permeability (P_m) is a function of the membrane diffusion coefficient (D_m), membrane partition coefficient (K_m), and the thickness of the membrane (L), as follows (17):

$$P_m = D_m \cdot K_m / L$$

Intestinal membrane thickness is equivalent to the enterocyte cellular membrane, a lipid bilayer according to the fluid-mosaic model. However, the intestinal barrier in situ also includes an unstirred water layer that may have differential effects on drug absorption. Two of these three factors, D_m and K_m , are strongly influenced by the lipophilicity of the solute.

The ability of NCE to permeate cell membranes by passive diffusion is primarily dependent on its partitioning into the membrane lipid bilayer. The most frequently used PC parameter for predicting cellular permeability is Log P , but the correlation between P_m and Log P has yielded mixed

results for diverse molecular structures. In general, more lipophilic compounds have higher cellular permeability coefficients (30). In fact, a plot of permeability vs. lipophilicity has been shown to be sigmoidal when the relationship is plotted for compounds grouped by molecular weight (31). The observed permeability plateau at high lipophilicity values is likely due to a stagnant diffusion layer, where permeation is rapid and diffusion through the unstirred layer is rate limiting. This relationship has been demonstrated *in vivo*, where it was observed that the rate of disappearance of drugs from a rat intestinal loop preparations correlated well with lipophilicity, up to a Log P value of +3 (32). This relationship has been confirmed *in vitro*, where the plateau occurs when Log P values exceed +3.5 (33). The tailing effect at low lipophilicity values is due to uptake of small hydrophilic compounds via the paracellular pathway, which has a much lower capacity due to size exclusion effects. The intervening steep part of the curve represents the critical range for oral absorption (28).

As described previously, immobilized artificial membrane chromatography has been used reasonably successfully to evaluate lipophilicity by measuring k_{IAM} (capacity factor) as a surrogate for the membrane partition coefficient K_m . This method has also been used as a simple and high-throughput method for predicting membrane permeability. Although the correlation between k_{IAM} and Log P is only moderately good for structurally diverse compounds ($r = 0.520$), it is better for fraction absorbed in the perfused rat intestinal model ($r = 0.791$) and for the apparent permeability (P_{app}) in enterocyte monolayers. In an excellent example of the rapid evolution of new higher-throughput assays, Kansy et al. (19) markedly increased the throughput of the standard IAM chromatography by developing the parallel artificial membrane permeability assay (PAMPA). Their objective was to classify passively transported NCE with respect to the transcellular route of absorption. A hydrophobic filter support material was coated with lecithin in the presence of an inert solvent in a 96-well plate format and the flux of solutes through the stable lipid bilayer was measured. The concentration of the compounds in the receiver side was determined by simultaneous UV measurement at six different wavelengths using a microplate spectrophotometer. A reference solution defining equilibrium conditions was used as an internal standard. Well-absorbed compounds (human absorption = 70–100%) exhibited a flux of 23–100%, intermediate-absorbed compounds (30–70%) exhibited 5–25% flux and poorly absorbed compounds (0–30%) exhibited <5% flux. Approximately 80% of the compounds would be correctly predicted with respect to human absorption with this method. Table 3 illustrates results obtained in our laboratory with this assay. The final test concentration is 50 μ M, the incubation time is 18 hr and analysis is by LC-MS/TOF.

The role of aqueous solubility in membrane permeability and drug absorption *in vivo* was somewhat overlooked until a drug classification

Table 3 Ranking of Existing Medicines for Transcellular Permeability with the Parallel Artificial Membrane Permeability Assay in 96-Well Format

Compound	Permeability
Atenolol	0.5 ± 0.7
Cimetidine	3.2 ± 0.2
Nadolol	3.3 ± 0.3
Doxorubicin	12.4 ± 3.7
Erythromycin	40.4 ± 6.4
Metoprolol	50.3 ± 7.1
Propranolol	84 ± 18
Verapamil	100.3 ± 4.6
Imipramine	100.3 ± 3.1

Values are mean percent flux $\pm$ SEM.

system based on permeability and solubility was proposed as a basis for establishing in vitro–in vivo correlations (34). Essentially, there is a good correlation between the extent of absorption in humans with enterocyte permeability in vitro (see below), but the strength of the association is limited by aqueous solubility. This relationship has been confirmed for a group of nine existing medications, among which was represented a wide range of values for aqueous solubility (0.03–465 mg/mL), lipophilicity (Log $P = -0.8$ to $+3$) and enterocyte monolayer permeability coefficients ($P_{app} = 10^{-7}$ to 4×10^{-5} cm/sec) (35).

The molecular size of NCE also affects their membrane permeability. Molecular size is a component of lipophilicity and the diffusion coefficient D_m in biological membranes and through membrane pores. Molecular size is frequently described by molecular weight (28). Therefore, two molecular size effects exist: the larger the molecular size of a compound, the smaller becomes its permeability coefficient through the membrane pores and the smaller the diffusion coefficient through the lipid part of biological membranes (30). Finally, an excessive number of hydrogen bond donor groups included in a new medicinal compound impair the membrane permeability (7,36). This influence is accounted for quite well in the measurement of Log P , because of the similarities in hydrogen bonding between lipid membranes and water-saturated octanol. The combination of high molecular weight and high Log P is observed in very few existing medications ($\sim 1\%$), but these characteristics appear to be enhanced in the leads from high-throughput screening (7).

Lipinski et al. developed and published a practical method to predict the permeability of NCE across the intestinal membrane (7) and flag unsuitable compounds. It incorporates many of the physicochemical factors

described previously in this chapter. It is commonly called the “rule of five” and it states that poor absorption or permeation is more likely when

- There are more than five hydrogen bond donors (the sum of OHs and NHs).
- The molecular weight is greater than 500.
- The Log *P* is over 5.
- There are more than 10 hydrogen bond acceptors (the sum of Ns and Os).

Compound classes that are substrates for biological transporters are exceptions to this rule.

3.2.2. Cell Monolayers

The measurement of permeability alone is difficult to conduct *in vivo*. Oral absorption data are frequently difficult to interpret because of numerous factors that affect the process (dissolution rate, first-pass metabolism, excretion). However, two low-throughput models were developed for intestinal absorption studies. Animal models are based on *in situ* isolation of intestinal loops, in which the disappearance of drug from the loop, or appearance in the blood, is monitored. Alternately, an intestinal segment is isolated and mounted in an Ussing chamber. The segment is placed between donor and receiver compartments. These have been used to characterize several factors that determine the transepithelial movement of drugs (37) and the results from each method correlate well with one another and with the fraction absorbed in human subjects (38).

The increasing pressure to screen many NCE for intestinal permeability motivated the search for new *in vitro* models. Caco-2, a human colorectal carcinoma cell line, was first used to study glycogen metabolism (39). Shortly thereafter, it was noted that Caco-2 cells were unique among many similar cell lines (e.g., HT-29 cells). After they reach confluence in culture, Caco-2 cells spontaneously differentiate into polarized, columnar cells that are more representative of the small intestine. They exhibit well-developed microvilli and a polarized distribution of brush-border enzymes, and their electrical properties resemble colonic crypt cells (40–42). However, it was not until 1989 that a report was published suggesting that Caco-2 cell monolayers could be used as a model to predict intestinal permeability (43). Similarity in uptake and barrier properties between this system and the small intestine epithelial layer were observed. Almost immediately, a series of six β -blocking drugs were tested with Caco-2 monolayers for permeability. The transport rates for four of the six compounds were similar in the Caco-2 model and in a rat intestinal loop model.

In fact, the Caco-2 model has been used with increasing frequency during the past 10 years as an *in vitro* surrogate for human intestinal

permeability. This is based on research establishing the relationship between the apparent permeability coefficient (P_{app}) in Caco-2 monolayers, where

$$P_{app} = dM_R/dt \cdot 1/A \cdot (C_{D,mid} - C_{R,mid})$$

and the fraction of the oral dose absorbed in human subjects (F_a). In the relationship above, dM_R/dt is the appearance rate of the test compound in the receiver side, A is the surface area of the cell monolayer, $C_{D,mid}$ is the mid-point concentration of the test compound in the donor side and $C_{R,mid}$ is the mid-point concentration of the test compound in the receiver side. In a rapid follow-up study, 20 well-known drugs (Log $D = -4.5$ to $+3.48$) with different structural properties were tested in Caco-2 monolayers (44). The investigators concluded that when a drug was completely absorbed in humans, P_{app} exceeded 2×10^{-6} cm/sec and when drugs were absorbed in humans less than 100%, $P_{app} = 0.1$ to 1×10^{-6} cm/sec. Since 1991, the fundamental hyperbolic relationship between fraction absorbed (F_a) and P_{app} in vivo has been clarified by a series of small studies. Co-cultures of absorbing Caco-2 cells and mucus-secreting HT29-MTX cells have been used to simulate the unstirred water layer. A good prediction of F_a in humans was attained by separating the passively transported drugs ($n = 15$) into two groups—well-absorbed compounds ($P_{app} > 10^{-6}$ cm/sec) and drugs that exhibit 40–70% absorption ($P_{app} < 10^{-6}$ cm/sec) (45). A strong correlation was observed between human absorption in vivo and P_{app} for a heterogenous collection of existing drugs ($r = 0.950$, $n = 35$). The authors observed that if F_a was 0–20%, P_{app} was less than 10^{-6} cm/sec, if F_a was 20–70%, then P_{app} was between 10^{-7} and 10^{-6} and if F_a was 70–100%, then P_{app} exceeded 10^{-6} . In this group, the range of $M \log P$ values was -4.91 to $+3.88$ (46).

The Caco-2 model appears to be a reasonable and reliable method to predict the fraction of intestinal absorption in humans, and attempts to improve it continue. One sub-clone of Caco-2, TC-7, has been identified by higher levels of expression of sucrase isomaltase and glucose transporters and increased taurocholic acid transport, compared to the parental Caco-2 cell line. The activity of phase II enzymes (glucuronosyl-transferase and glutathione transferase) appears to be similar to human jejunum, and higher than in Caco-2 cells (47). In addition, TC-7 is more homogenous in terms of cell size and confluence is achieved earlier than Caco-2 cells, due to a shorter doubling time (26 vs. 30 hr). Furthermore, P-glycoprotein-mediated cyclosporin efflux was less strongly expressed in TC-7 cells than in Caco-2, thereby allowing less complicated measurement of permeability. A threshold for absorption in humans, 2×10^{-4} cm/sec, above which 100% oral absorption is very nearly equivalent to a P_{app} value observed in Caco-2 monolayers of 2×10^{-6} cm/sec (48). These studies have demonstrated the importance of analyzing the permeability of NCE in relation to a set of several reference compounds exhibiting a large range of permeability and for which

absorption from an orally administered dose in humans is known (e.g., propranolol). The major reason for employing reference standards in these assays is the observed variation in P_{app} values among testing facilities, which is primarily a result of differences in culture conditions. In addition, a relatively hydrophilic reference standard is included as an index of monolayer integrity (e.g., mannitol).

Despite recent advances in this model, human colorectal adenocarcinoma cell permeation studies are laborious and therefore not suited to high-throughput measurements of intestinal permeability. The Caco-2 assay remains a relatively low-throughput method, due in part to the limitations of its 21-day growth period and regular maintenance feeding requirements. Proprietary culture conditions that accelerate differentiation to three days become costly for the purpose of screening large series of compounds (49). Until recently, the functional lower limit on the area of cell monolayers has restricted this assay to 6-, 12-, or 24-well Transwell plates, in order to accommodate low-permeability compounds. Typically, medicinal compounds are tested at an initial donor-side concentration of 10–50 μM . The rate at which samples are analyzed may be sustained by pooling aliquots from the donor wells and from the receiver wells, except from wells in which isobaric compounds are being tested. Table 4 illustrates the results of evaluating the permeability (A–B) of existing medications, tested in duplicate, using Caco-2 monolayers in a 96-well format. The value of percent recovery is the method used to determine the validity of an experiment, that is, data from an experiment with a recovery of less than 50% are considered unreliable. In our laboratory, a compound is considered to be highly permeable (well-absorbed) if the value of P_{app} is greater than 1×10^{-5} cm/sec. The final test concentration is 30 μM and analysis is conducted with LC-MS on a triple quadrupole mass spectrometer. A caveat to using this method of evaluating permeability/absorption is that there is no unified cell culture or experimental protocols and consequently, the inter-laboratory variability in the value of P_{app} for any given compound is frequently significant. The criteria to distinguish well-absorbed compounds from poorly absorbed compounds need to be established at every location where the assay is conducted.

In an attempt to further reduce time, cost, and effort, monolayers of the Madin-Darby Canine Kidney (MDCK) epithelial cell line have also been investigated as an *in vitro* model to measure the relative permeability of NCE. This approach was suggested by Cho et al. (50) and MDCK monolayers were first tested on antimicrobials (51). MDCK cells reach confluence after three days because they can be seeded at high density (650,000 cells/cm²). Like Caco-2 cells, MDCK differentiate into columnar epithelium after reaching confluence and they form tight junctions on semi-permeable membranes. This manipulation does not work to reduce culture time for Caco-2 because cells seeded at high density display high permeability for lucifer yellow by day 3, typical of poor tight junction integrity (52). Irvine

Table 4 The Permeability of Existing Medicinal Compounds in Caco-2 Monolayers

Compound	A–B				B–A				B–A/ A–B Ratio	Literature values			
	P_{app}		Recovery (%)		P_{app}		Recovery (%)			A–B	B–A	B–A/ A–B	Human absorption (%)
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM					
Ranitidine	4	1	88	5	3	0	99	9	0.83	0.49 ^a , 1.4 ^b	1.95 ^b	1.35 ^b	55 ^a
Erythromycin	4	1	91	3	10	1	110	10	2.52	3.73 ^a			35 ^c
Doxorubicin	5	1	81	16	5	2	87	12	0.95	0.16 ^a , 1.5 ^b	0.97 ^b	0.67 ^b	5 ^a
Atenolol	6	2	88	3	2	1	110	10	0.37	1.2 ^c , 3 ^h			40–70 ^c
Nadolol	7	1	85	4	4	1	98	6	0.58	3.88 ^a , 2.2 ^h			32 ^a , 15 ^h
Cimetidine	8	3	83	2	9	1	97	4	1.11	0.74 ^a , 3 ^f			62 ⁱ
Verapamil	27	4	101	6	66	11	105	11	2.46	59 ^b	68.7 ^b	1.16 ^b	
Imipramine	32	8	92	5	55	8	90	12	1.74	14 ^a			99 ^a
Propranolol	37	6	90	5	52	3	94	8	1.39	41.9 ^a , 49.6 ^b , 34.4 ^c , 37.6 ^d , 86 ^f	51.4 ^b	1.04 ^b	93 ^a
Metoprolol	47	4	124	6	80	12	112	6	1.71	23.7 ^a , 18 ^c , 23.6 ^d			95 ^a

Well absorbed: $P_{app} > 10 \times 10^{-6}$ cm/sec. Poorly absorbed: $P_{app} < 10 \times 10^{-6}$ cm/sec. P-gp substrate, ratio >2 . Not a P-gp substrate: ratio <2 .

^aZhu C, et al. Eur J Med Chem 2002; 37:399–407.

^bSee Ref. 60.

^cSee Ref. 48.

^dTannergren C, et al. Pharmazie 2001; 56(4):337–342.

^eSee Ref. 46.

^fSaha P, et al., Eur J Pharm Biopharm 2000; 50:403–411.

^gChong S, et al., Pharm Res 1997; 14(12):1835–1837.

^hMarkowska M, et al. J Pharm Tox Meth 2001; 46:51–55.

ⁱSee Ref. 35.

et al. (51) tested 55 compounds, with known permeability values, in Caco-2 and MDCK monolayers. Their results suggested that P_{app} values measured in MDCK monolayers correlated well with P_{app} values from parallel Caco-2 experiments ($r^2 = 0.79$). In addition, Spearman's rank correlation coefficient for MDCK P_{app} and human absorption was 0.58, compared with 0.54 for Caco-2 P_{app} and human absorption. These results suggest that, under certain conditions, MDCK monolayers may be another useful tool for rapid permeability screening.

Another approach to increasing the throughput of permeability screening is the use of a single enterocyte monolayer to screen a mixture of NCE. Taylor et al. (53) screened six arbitrary mixtures of 24 physico-chemically diverse, *N*-substituted glycine peptoids. They used this technique to study structure–transport relationships. They added a unique methodological twist by analyzing the donor and receiver compartments for permeability and the receiver compartment for pharmacological activity. This process of coupling screens for therapeutic activity and permeability is very representative of the type of innovation possible.

A major challenge for measuring permeability of libraries is the need for sensitive quantitative analytical techniques. Sensitivity is dictated by the solubility in the donor medium and the achievable concentration of transported compounds in the basolateral receiver compartment. It has been estimated that the application of LC-MS in single-ion mode to these permeability assays improves detection 1000-fold over HPLC and enhances selectivity over HPLC/UV, which is extremely important in analyzing mixtures (54). Most recently, a report was published detailing the permeability screening of a combinatorial library containing 375,000 peptides (55). This mammoth task was accomplished testing a series of 150 pools, each containing 2500 tripeptide sequences. The NCE in the receiver compartment were separated by capillary high-performance liquid chromatography and analyzed by LC-MS/MS to identify structures. To discriminate between isobaric structures, several compounds were re-synthesized and re-tested individually.

3.2.3. P-glycoprotein, Drug Absorption, and Drug Elimination

The development of very potent and selective medications has implications on the dose size and dosing frequency of dosing. Ideally, medicines require small and infrequent doses. Under these circumstances, the role of the ATP-binding cassette anti-porter P-glycoprotein (P-gp) may become very important in determining pharmacokinetic variables such as bioavailability. As detailed in [Chapter 8](#), this protein is expressed in the intestinal epithelium, liver, kidney, and the blood–brain barrier and it is capable of restricting passage across these cellular barriers and influencing the absorption, distribution, and elimination of many drugs. It has a very broad substrate specificity that overlaps with that of cytochrome P4503A4 in many instances.

Therefore, it has become important to determine very early on if compounds are subject to being influenced by P-glycoprotein. Several methods are available to do so.

Until recently, the investigation of interactions between NCE and P-glycoprotein was typically conducted during pre-clinical testing, if at all. It has focused on the use of a low-throughput Caco-2 cell monolayer model to determine the mucosal-to-serosal (apical-to-basal) efflux of development candidates relative to existing medications, which are used as positive and/or negative controls, or in the presence or absence of widely used inhibitors such as verapamil (56). However, new models for higher-throughput assays have been developed to provide information on P-gp interactions during drug discovery. One of these monitors the NCE-stimulated ATPase activity of P-gp in isolated cell membranes, measuring the appearance of inorganic phosphate by a colorimetric reaction (57–59). Figures 1–3 illustrate the differences we have observed in the concentration-dependant ATPase activity

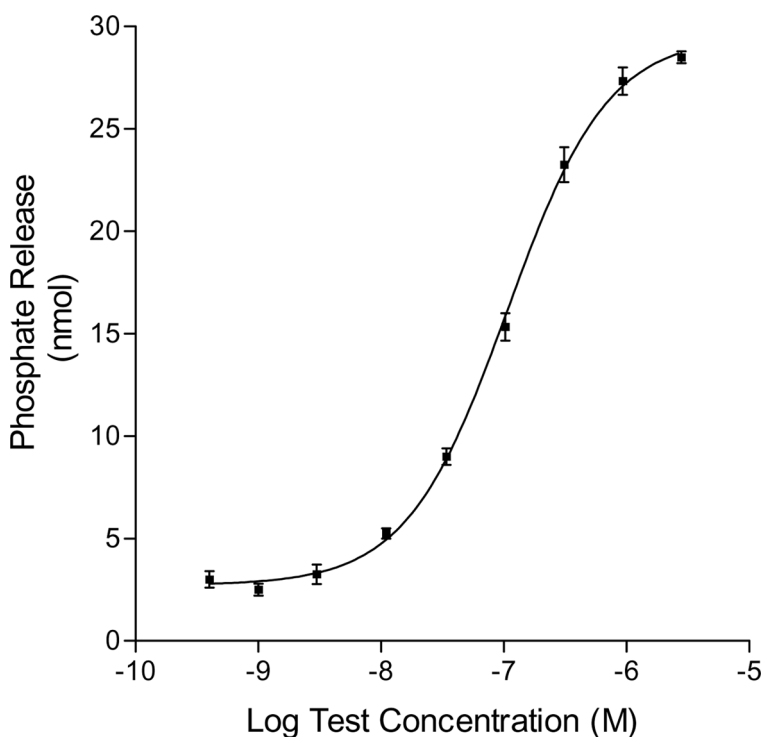


Figure 1 The ATPase activity of ritonavir in a membrane-based assay for P-glycoprotein interaction. Membranes isolated from cells expressing P-glycoprotein (250 $\mu\text{g}/\text{mL}$) were incubated with ritonavir in Tris–MES buffer (final volume = 60 μL) for 20 min at 37°C.

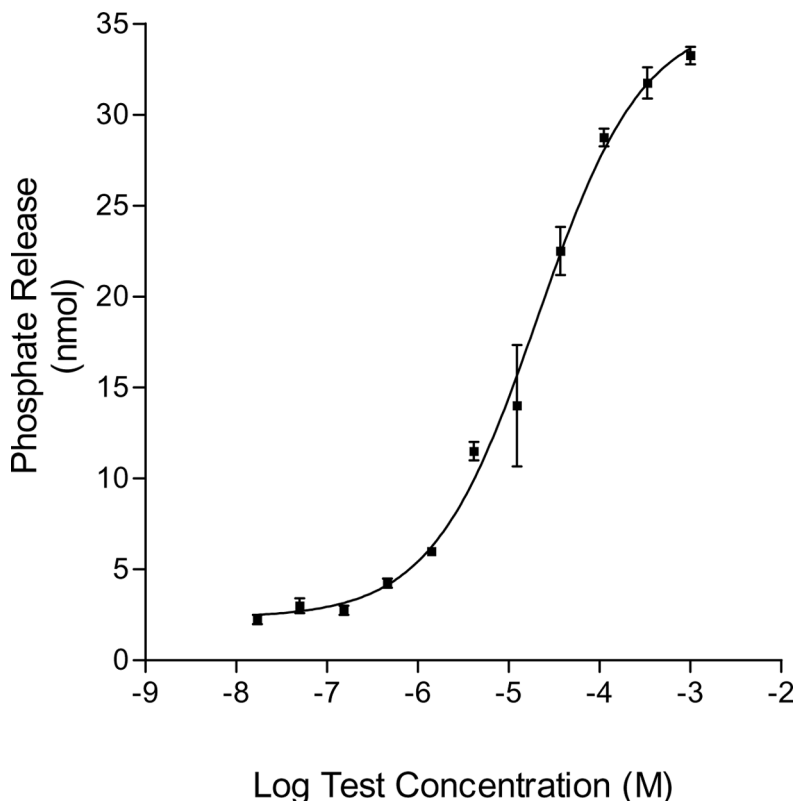


Figure 2 The ATPase activity of verapamil in a membrane-based assay for P-glycoprotein interaction. Membranes isolated from cells expressing P-glycoprotein (250 $\mu\text{g}/\text{mL}$) were incubated with verapamil in Tris–MES buffer (final volume = 60 μL) for 20 min at 37°C.

stimulated by ritonavir, verapamil or nifedipine respectively. Note the 1000-fold difference between ritonavir and verapamil with respect to the concentration at which the maximum effect was measured.

Unfortunately, some compounds identified as substrates in the ATPase assay do not appear to undergo significant transmembrane transport in Caco-2 monolayers. This is true of midazolam, which has high passive permeability (60), leading to fast transcellular flux that may overcome P-gp-mediated efflux (56,61). Conversely, some medicines previously identified as substrates in the Caco-2 model (e.g., vincristine, colchicine) fail to stimulate ATPase activity (62). A cell-based assay developed for this purpose involves the use of fluorescent substrates (e.g., calcein AM or rhodamine 123), where intracellular accumulation of the parent compound, or a fluorescent metabolite, is caused by the inhibition of P-gp by NCE (56,60). This

result is then compared to a standard positive control, such as nicardipine. These new assays appear to be suitable for high-throughput screening during lead selection, but they may be used to determine only if NCE interact with P-gp, not whether they are inhibitors or substrates (61). Therefore, neither of the assays alone may be useful during lead optimization, when minor differences between structurally similar NCE may become critical in selecting a development candidate. In fact, the use of all three assays has resulted in the classification of verapamil as a non-substrate, a substrate and an inhibitor (56,63,64).

Therefore, it may be very difficult to classify new compounds as inhibitors, non-transported substrates or substrates with a single assay, because different models/assays and test conditions frequently yield different results. It is becoming clear that efflux or inhibition data from P-gp interaction studies conducted in Caco-2 monolayers or other cultured cells expressing P-gp (e.g., human renal proximal tubule epithelial cells) depends on the substrate selected. In fact, the particular cell type chosen for screening may influence the kinetic properties of P-gp. Disparities arise not only from differences in assay conditions, but also classification criteria and nomenclature (59). Furthermore, assay reproducibility may be poor as typical testing concentrations (20–50 μM) frequently exceed the solubility of many NCE (61) in cell culture media. Therefore, it has been recommended that high-throughput screening for P-gp interactions using membrane- or cell-based assays during lead selection should be combined with an assay that can distinguish between substrates and inhibitors, even if the results from a fluorescent assay are negative (61).

In an attempt to clarify this confusing situation, Polli et al. (59) have developed a rather complex classification system, based on the results of screening a variety of medicinal compounds in Caco-2, ATPase, and Calcein AM inhibition assays. Category I compounds (possible inhibitors) are not effluxed in standard Caco-2 assay, nor do they stimulate ATPase activity (e.g., testosterone). However, they cause an accumulation of Calcein AM in a cell-based assay by inhibiting P-gp. Compounds placed in Category IIA (non-transported substrates) are not effluxed in Caco-2 monolayers, but they test positive in the ATPase and Calcein AM assays (e.g., verapamil). Compounds falling into Category IIB are considered transported substrates. Category IIB₁ compounds test positive in the efflux assay but negative in the ATPase and Calcein AM assay (e.g., vincristine). Category IIB₂ compounds are effluxed in Caco-2 and positive in the ATPase assay, but they are negative in the Calcein AM assay (e.g., erythromycin). Category IIB₃ compounds are effluxed in the Caco-2 assay and positive in the Calcein AM assay, but they do not stimulate ATPase activity (e.g., cyclosporin). Compounds in category IIB₂ and IIB₃ are considered transported substrates. Table 4 also illustrates the use of Caco-2 monolayers to determine if a compound interacts with P-gp. Typically, compound should be considered a P-gp substrate when the value of P_{app} in the basal-to-apical

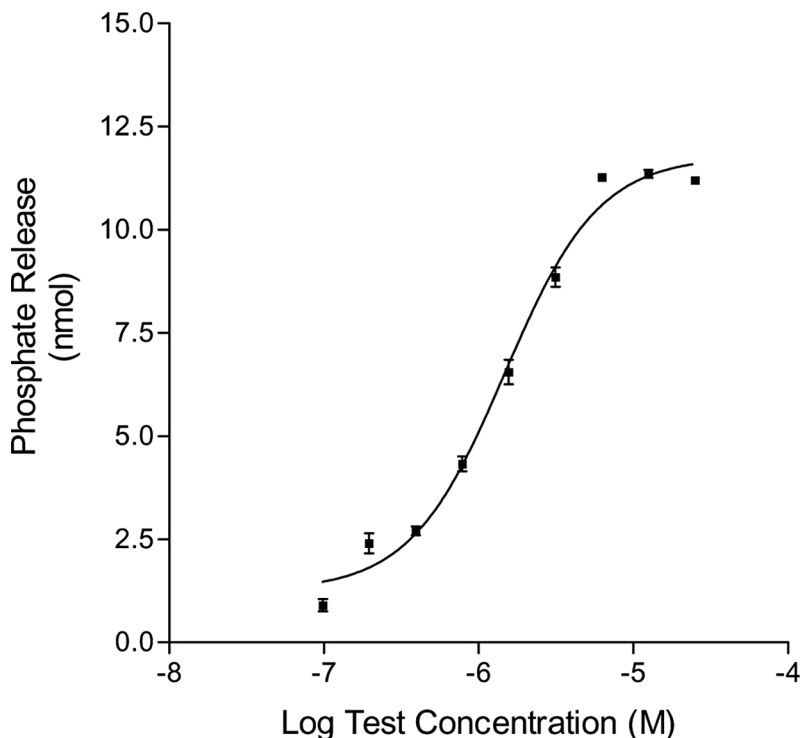


Figure 3 ATPase activity of nicardipine in a membrane-based assay for P-glycoprotein interaction. Membranes isolated from cells expressing P-glycoprotein (250 $\mu\text{g}/\text{mL}$) were incubated with nicardipine in Tris-MES buffer (final volume = 60 μL) for 20 min at 37°C.

(B–A) direction exceeds the value of P_{app} in the apical-to-basal (A–B) direction by a factor of 2 or more (Ron Borchardt, personal communication).

3.3. Hepatic Metabolism

3.3.1. Metabolic Stability and Intrinsic Hepatic Clearance

A recent review, published in honor of Dr. AYH Lu, recounts the evolution of drug metabolism science in the 20th century. The essay includes a brief description of current processes and low-throughput in vitro procedures typically used in drug metabolism investigations (65). Reviews detailing the application of these in vitro tools and techniques have also been published recently (66,67).

Historically, the study of pharmacokinetic factors (i.e., intestinal absorption, pre-systemic metabolism and clearance) have been delayed until pre-clinical development. But these factors can have a profound impact on

the duration and intensity of pharmacological effects by altering the bioavailability of medicinal compounds. Short duration of action renders it impossible to provide a patient with a convenient dosage regimen that encourages compliance. So human pharmacokinetic characterization is being shifted from pre-clinical development to (late) discovery.

It is generally desirable to design a safer drug that undergoes predictable metabolic inactivation or undergoes little or no hepatic metabolism. This simplifies the pharmacokinetics due to a lack of inter-individual variation observed when hepatic drug-metabolizing enzymes, particularly cytochrome P450s, are involved. In addition, drugs which undergo extensive first-pass metabolism are potentially susceptible to drug interactions (68). Although metabolically inert compounds are highly desirable candidates, the versatility of hepatic drug-metabolizing enzymes presents quite a challenge to achieving this goal (69). Examples of poorly metabolized drugs include the ACE inhibitor lisinopril and the β -blocker atenolol. This characteristic is attributable to their relatively low lipophilicity.

The advent of combinatorial chemistry presents a formidable challenge to metabolism scientists to devise reliable, higher-throughput methods of assessing metabolic stability and potential metabolic drug interactions. In a practical sense, the objective is to prevent drug metabolism studies from becoming a bottleneck in drug discovery. The target capacity for these drug metabolism screens is in the order of hundreds of compounds per week (70). The search for systems that can meet these requirements has focused on automation and miniaturization of existing methods, but any improvements in throughput are worthless unless they are supported by rigorous and continuing validation of overall performance.

In vitro models for the study of drug metabolism are probably the best established of the lead selection/optimization sciences discussed in this chapter (56,57). They have been used for two decades in pre-clinical metabolism studies to supplement pharmacokinetic and safety assessments in vivo. Liver S9 fraction and microsomes are the most widely used model for these experiments, but human and non-human hepatocytes and liver slices have become readily available for these purposes.

Hepatic metabolism continues to be a major factor affecting the progression of potential lead compounds through pre-clinical and human clinical studies. During drug discovery, measurement of relative metabolic stability in vitro provides a rapid means of ranking a series of molecules in the absence of factors such as absorption, serum protein binding, and blood flow. In the experimentally simplest procedure, the extent of metabolism is determined from the ratio of parent compound remaining in the test sample to that in the control. Alternately, NCE can be ranked by the initial rate of the disappearance of the parent compound (V_0), the in vitro half-life ($t_{1/2}$) or the intrinsic clearance (60,71). These protocols generally require a

larger number of samples per compound, and consequently more bioanalytical and data management resources.

In vitro metabolism studies now conducted in drug discovery may also be used to predict certain pharmacokinetic variables, because frequently the failure of candidate compounds in the clinic is associated with poor pharmacokinetic behavior. However, these predictions are based on relatively elaborate experiments that are not easily adapted to rapid compound selection. Well before high-throughput profiling was introduced, it was observed that, in rats under first-order conditions, the contribution of hepatic drug-metabolizing enzymes may be estimated by the ratio of the Michaelis–Menten kinetic constants $V_{\max}$ and K_M , normalized to the amount of microsomal protein and scaled up to reflect liver microsomal protein content. This determination is equivalent to the intrinsic hepatic clearance of the drug (Cl_{int}). This value was then used to predict the extraction ratio (E_h) for several existing medications, where

$$E_h = Cl_{\text{int}} / (Cl_{\text{int}} + Q)$$

and Q is hepatic blood flow, derived from existing physiological databases. The value of E_h is related to another very important pharmacokinetic characteristic of orally administered medicines, bioavailability (F_a), where $F_a = (1 - E_h)$. A comparison between the predicted ratio, based on a microsomal model of hepatic elimination, with that determined directly in the isolated perfused liver suggested good agreement between the predicted and observed hepatic extraction ratios (72). At that point in time these results were probably considered of academic interest, but the metabolic screening of libraries of medicinal compounds renewed the interest in pharmacokinetic predictions based on simple protocols (73–75). In one comprehensive analysis of predictive human models (64), 12 methods were assessed for their utility in predicting Cl_{int} . The most useful methods in which in vitro metabolism data from human liver microsomes were scaled to in vivo clearance values yielded predicted clearance values that were, on average, within 70–80% of actual values. However, differences in Cl_{int} in vitro and Cl_{int} in vivo greater than fivefold have been observed (65). Furthermore, it appears that there are significant differences in the values obtained for Cl_{int} , and that these differences are related to the model selected for the evaluation (64,76).

An important assumption in initial studies of predictive models was that drug binding to incubation constituents would not have a significant impact on the scale-up of in vitro clearance data to in vivo clearance because of typically low protein concentrations in microsomal incubations compared to concentrations of protein in plasma. However, the degree of non-specific binding of NCE to microsomal protein and partitioning into microsomal lipids during incubations recently has been shown to influence the results of liver microsomal metabolic stability screening is (77–80). If this phenomenon exists for even a small proportion of medicinal compounds screened

each year, it could have a widespread impact on drug discovery because liver microsomal studies have retrospective importance for drug metabolism investigations *in vitro*. However, it is still not known if non-specific binding to microsomes and constituents of other *in vitro* models is characteristic of a particular subset of compounds or unique to each compound, or how the binding of specific drugs varies between *in vitro* models. When the Michaelis–Menten constants are used to estimate Cl_{int} , it appears that if non-specific binding reduces unbound drug significantly, K_M values are overestimated, because they are based on the nominal substrate concentration added to the incubation and not the free substrate available to bind to the enzyme.

It appears that the fraction unbound in the incubation matrix is highly dependent on the microsomal protein concentration. In one report, *in vitro* methods generally under-predicted intrinsic clearance *in vivo*, but these compounds were highly bound to plasma protein and all were lipophilic amines (69). Therefore, taking into account non-specific binding, intrinsic clearance may be estimated as follows:

$$Cl_{int} = 0.693 \cdot \text{liver weight/in vitro } t_{1/2} \cdot \text{liver in incubation} \cdot f_u$$

For human predictions, “liver weight” is 21 g per kg body weight, “liver in incubation” is grams liver per mL incubation matrix (derived from a value of 45 mg microsomal protein per gram liver). The fraction of unbound drug in the incubation (f_u) can be determined by equilibrium dialysis of microsomes against a physiological buffer.

Initial reports using *in vitro* metabolism data for the prediction of pharmacokinetic behavior have been followed by a tide of very revealing reports describing direct comparisons between rat liver microsomes and isolated rat hepatocytes (63,81–83), investigating metabolism using rat liver slices (84–86) and detailing comparisons of all three models (66,87). Most recently, these inquiries have focused on the contribution of some of the principal microsomal cytochrome P450 enzymes involved in drug metabolism (88,89). These studies have revealed some model-specific and drug-related artifacts that are probably responsible for the kinetic differences observed between liver microsomes, isolated hepatocytes, and liver slices. Differences have been reported for a small set of reference compounds, including tolbutamide, phenytoin, caffeine, diazepam, ethoxycoumarin and dextromethorphan. These mature medicines have been well characterized with respect to their pharmacokinetic behavior *in vivo*. However, significant compound-related effects on predictions have also been demonstrated in these models (74). For example, studies have consistently shown that, after appropriate consideration of experimental conditions (77), predictions of intrinsic clearance from isolated hepatocytes are closer to *in vivo* values than those from microsomal studies for phenytoin, but not for tolbutamide (71). This phenomenon may be rationalized by either end-product inhibition

in microsomal incubations or differences in non-specific binding to microsomal components. Furthermore, significant differences have been observed between microsomes and hepatocytes with respect to metabolite profile that are unrelated to differences in nominal drug concentration (72). Liver slices appear to under-predict $V_{\max}$, over-predict K_M and, therefore, under-predict intrinsic clearance, relative to isolated hepatocytes (75,76). This effect may be attributed to poor diffusion of the substrate into all cells in a slice or restricted oxygenation leading to compromised metabolic function. Finally, the metabolism of a number of compounds by CYP3A4 by liver microsomes and hepatocytes does not exhibit classic Michaelis–Menten kinetics, but displays sigmoidal kinetics (79). Consequently, intrinsic clearance cannot be calculated for these drugs due to the lack of a first-order region in their kinetic profiles. A suitable method has yet to be identified to allow these results to be scaled to predict in vivo clearance. The effect of this circumstance could be enormous, considering the large proportion of existing medications that are metabolized by CYP3A4. Figures 4–7 illustrate the determination of intrinsic clearance in rat or human liver microsomal incubations, and the species differences that frequently occur. The value of intrinsic clearance, Cl_{int} , is proportional to the slope of the regression line. This assay also yields information on the metabolic stability of NCE at 30 min.

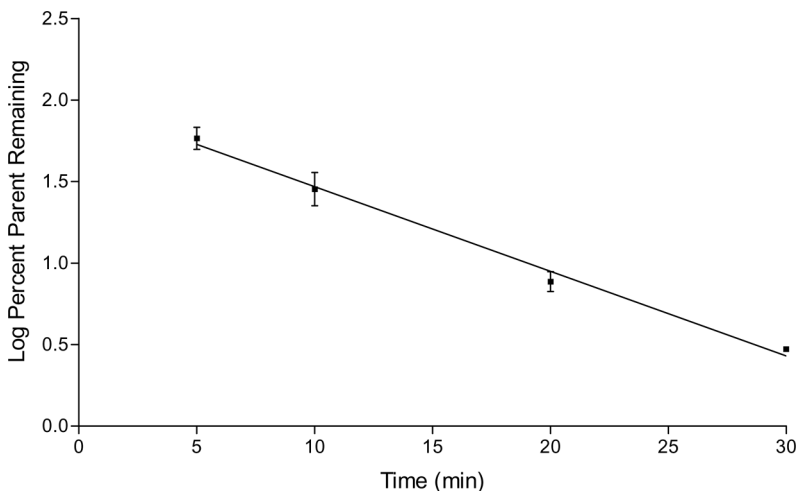


Figure 4 The intrinsic clearance of trazadone, a high clearance compound, determined in pooled rat liver microsomal incubations. Rat liver microsomes (1 mg/mL) were incubated with trazadone (10 μ M) and NADPH (10 mg/mL) in potassium phosphate buffer (100 mM, pH 7.4) for 30 min at 37°C. Aliquots (45 μ L) were taken at 0, 5, 10, 20 and 30 min and reactions were stopped with 100 μ L of cold acetonitrile. $Cl_{\text{in}} = 54.3 \pm 7.5$ mL/min/kg (mean $\pm$ SE).

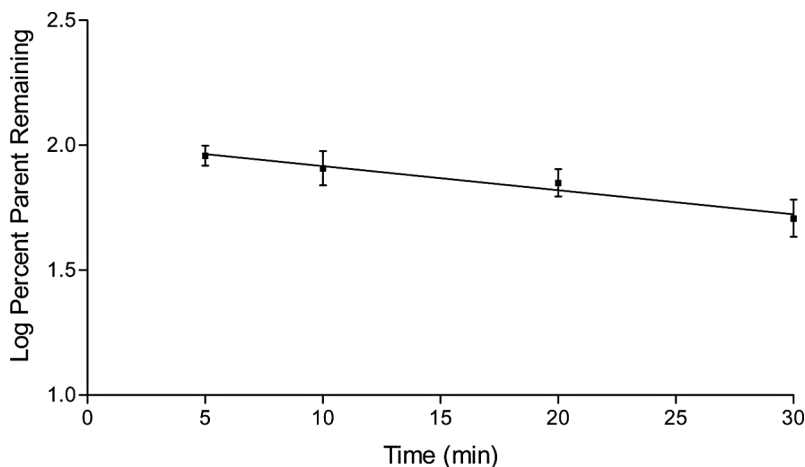


Figure 5 The intrinsic clearance of desipramine, a low clearance compound, determined in pooled rat liver microsomal incubations. Rat liver microsomes (1 mg/mL) were incubated with desipramine (10 μ M) and NADPH (10 mg/mL) in potassium phosphate buffer (100 mM, pH 7.4) for 30 min at 37°C. Aliquots (45 μ L) were taken at 0, 5, 10, 20 and 30 min and reactions were stopped with 100 μ L of cold acetonitrile. $Cl_{in} = 7.5 \pm 3.2$ mL/min/kg (mean $\pm$ SE).

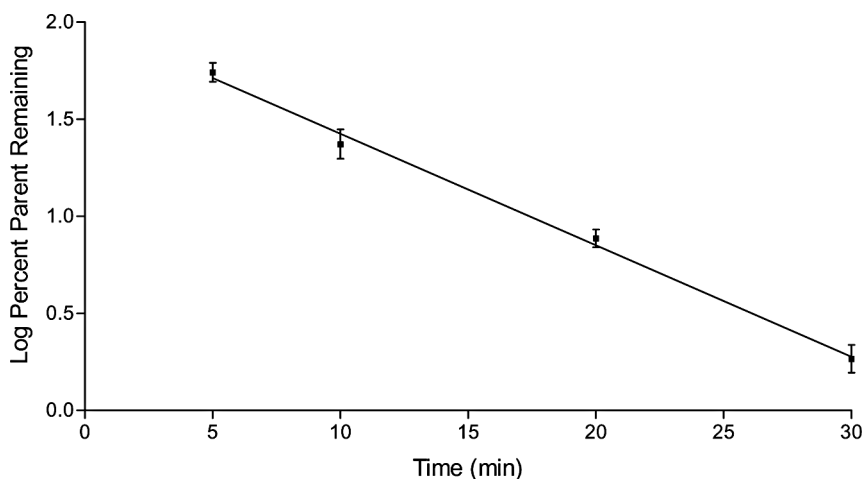


Figure 6 The intrinsic clearance of trazodone, a high clearance compound, determined in pooled human liver microsomal incubations. Human liver microsomes (1 mg/mL) were incubated with desipramine (10 μ M) and NADPH (10 mg/mL) in potassium phosphate buffer (100 mM, pH 7.4) for 30 min at 37°C. Aliquots (45 μ L) were taken at 0, 5, 10, 20 and 30 min and reactions were stopped with 100 μ L of cold acetonitrile. $Cl_{in} = 8.7 \pm 1.2$ mL/min/kg (mean $\pm$ SE).

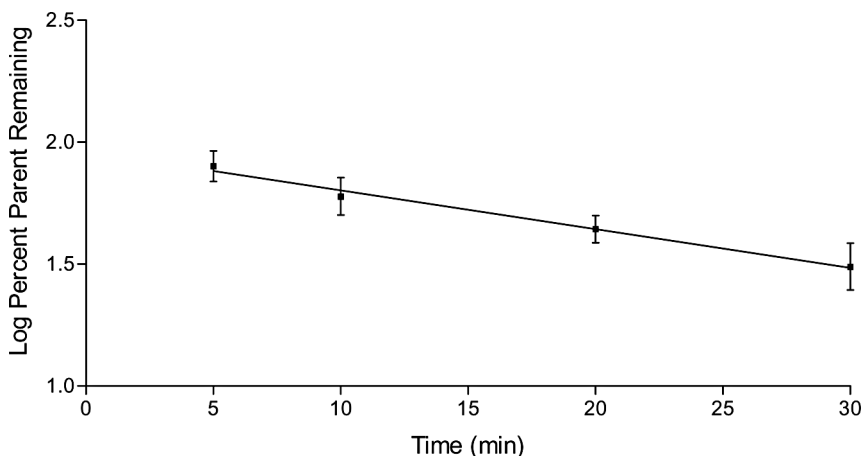


Figure 7 The intrinsic clearance of carbamazepine, a low clearance compound, determined in pooled human liver microsomal incubations. Human liver microsomes (1 mg/mL) were incubated with desipramine (10 μ M) and NADPH (10 mg/mL) in potassium phosphate buffer (100 mM, pH 7.4) for 30 min at 37°C. Aliquots (45 μ L) were taken at 0, 5, 10, 20 and 30 min and reactions were stopped with 100 μ L of cold acetonitrile. $Cl_{in} = 1.7 \pm 0.7$ mL/min/kg (mean $\pm$ SE).

The limited results of model-comparison studies may not be entirely applicable to new medicinal compounds arising from a combinatorial library and selected with high-throughput screening against a pharmacological target. However, all three models can be applied to ranking a series of compounds by V_{max} , K_M , and Cl_{int} (90) or to flag NCE having disadvantageous metabolic characteristics (analogous to Lipinski's "rule of five" used for permeability assessments). Unfortunately, many predictive protocols remain proprietary. Experimental constraints, such as the preparation and culture of hepatocytes and slices, and the associated analytical and informatics requirements, limit the usefulness of these methods in high-throughput screening, but they can be adapted to 48- and 96-well plates, or to a flow-through system. For example, rat liver microsomes (RLM) have been used to determine the extent of metabolism and to identify the major oxidative metabolites of imipramine (91). RLM were trapped in a stirred ultrafiltration chamber fitted with a 100 kDa molecular weight cut-off membrane. A continuous flow of ammonium acetate buffer was pumped through the chamber and into a triple quadrupole mass spectrometer equipped with an ESI interface. The substrate was flow injected through the chamber with NADPH and imipramine and its major metabolites were detected on-line. Only one chamber at a time was connected to the mass spectrometer. The method effectively screened 20–60 compounds per hour.

Regardless of the biological model and the experimental protocol selected for rapid metabolic screening, limitations on analytical resources to support metabolism screening can create a potential bottleneck in the pharmaceutical profiling process. HPLC/UV is probably adequate to detect most compounds in these assays (92), but the selectivity of LC-MS is generally preferred (93).

Practical experience has shown that miniaturization of *in vitro* assays is relatively straightforward, although the degree of agitation of the microtiter plates during incubation must be sufficient to ensure that the samples remain homogenous throughout the experiment (60). Typically, NCE are included in incubations at a final concentration of 1–10 μM and the final protein concentration is minimized to reduce non-specific binding (e.g., 0.5–1.0 mg/mL). Incubations are normally conducted in duplicate and each 96-well microtiter plate contains several reference substrates of varying metabolic stability (e.g., labetalol, verapamil, and terfenadine). Methods of ranking NCE by relative metabolic stability ($t_{1/2}$, V_0 , percent turnover) are widely used, but prediction of intrinsic hepatic clearance remains difficult and somewhat controversial.

3.3.2. Drug Interactions and Identification of Major Cytochrome P450 Enzymes

In the United States, the frequency of serious adverse reactions to drugs was recently estimated to be in the order of two million per year, of which 100,000 were fatal. A significant proportion of these incidents were observed in patients receiving multiple drugs (60). In fact, the morbidity and mortality resulting from a serious metabolic drug interaction between terfenadine and ketoconazole (94) caused scientists at the FDA and in the pharmaceutical industry to pay much more attention to the inhibition of hepatic drug-metabolizing enzymes (95). The problem of clinically relevant drug interactions arises from disturbances in pharmacokinetic behavior that raise the plasma concentration of one of the drugs above intended therapeutic levels. As the concentration of the parent drug rises, side effects appear as the selectivity of pharmacological action disappears. In the extreme situation, for example when the medicine exhibits a relatively low therapeutic index, serious adverse effects may appear. Therefore, metabolic drug interactions are also an issue of drug toxicity. Both intensity and duration of drug action can be affected. Medicines that are highly metabolized tend to be more prone to metabolic drug interactions and medications that are metabolized by several hepatic enzymes are less likely to cause clinically significant clinical interactions than drugs that are metabolized by one enzyme. Medicines that are metabolized extensively only by one of the polymorphically expressed microsomal P450 enzymes (i.e., CYP2C9, CYP2C19, CYP2D6) are also associated with a higher risk for drug-related toxicity in poor metabolizers (56,57). Due to the high cost of clinical investigations, there is a practical

limit to the number and scope of clinical drug interaction studies that can be performed. Inevitably, some significant interactions could remain untested before a drug is in widespread use.

In human liver, several microsomal cytochrome P450 enzymes act as principal drug-metabolizing enzymes (e.g., CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP2B6). Although each enzyme exhibits a degree of selectivity, members of the hepatic microsomal P450 superfamily generally exhibit broad and overlapping substrate specificity for a very wide variety of xenobiotics. However, the identity of the cytochrome P450 enzyme(s) involved in an oxidative pathway of drug metabolism can be determined by several methods (57). These protocols take advantage of the rapid commercialization of human liver products (e.g., liver microsomes, cryopreserved hepatocytes, recombinant enzymes, anti-P450 antibodies) during the past decade. In fact, by the mid-1990s, P450 identification studies became routine during the pre-clinical period because of the close association between drug interactions, pharmacokinetic behavior, and safety.

One direct approach to characterizing the inhibitory potential of a lead compound has been to determine and rank *in vitro* K_i values for compounds against enzyme-selective substrates, utilizing human liver microsomes (96). However, the relationship between *in vitro* K_i and the probability of adverse effects of an interaction is very difficult to predict. However, it may be possible to estimate the fractional decrement in clearance (FDCL) that could be expected *in vivo* by calculating a surrogate, the fractional decrement in velocity (FDV), *in vitro*. Under first-order conditions ($[S] \ll K_M$) or if the interaction is non-competitive

$$\text{FDCL} = V_0 - V_1/V_0$$

where V_0 is the velocity of the metabolic reaction in the absence of an inhibitor and V_1 is the reaction velocity in the presence of the inhibitor. However, these assays are typically labor-intensive and experimentally complex for the purposes of high-throughput pharmaceutical profiling. Therefore, the availability of heterologously expressed human liver microsomal cytochrome P450 enzymes and non-specific, high-affinity fluorescent substrates have enabled methods that can investigate the inhibitory potential of NCE in a high-throughput format (60). Most of these new substrates are derived from coumarin. In its most streamlined conformation in 96-well microtiter plates, this method facilitates the identification of NCE that have a high potential for metabolic drug interactions (97), as well as the principal P450 enzymes involved in the metabolism of certain series. The assay is typically used to screen a large number of compounds in duplicate at a single concentration (1–10 μM) against a concentration of fluorescent substrate equivalent to approximately twice the K_M . Each microtiter plate contains buffer controls, solvent controls, and several wells used to establish an IC_{50} curve for the reference inhibitor. Statistically, if one assumes these

assays exhibit a background inhibition of 10%, then inhibition becomes significant ($p < 0.05$) at 25%. Although criteria vary between laboratories, NCE that cause 80–100% inhibition at 10 μM are commonly re-tested to determine their IC_{50} . Inhibitory behavior is evaluated by using eight concentrations of NCE over three orders of magnitude and the IC_{50} is calculated with non-linear regression of the average fluorescence values at each NCE concentration. Experience has shown that the IC_{50} values determined with human liver microsomes and those derived with recombinant enzymes are rarely the same, primarily because these coumarin derivatives are not enzyme-specific substrates.

Screening for P450 inhibition with recombinant human enzymes requires the application of potent, if not highly specific, chemical inhibitors for each enzyme. The selectivity and potency of P450 enzyme inhibitors has been investigated in rat and human liver microsomes and microsomes containing a single human recombinant P450 enzyme. The information has been extremely useful in the effort to develop high-throughput cytochrome P450 inhibition assays (98–100). Currently there are a wide variety of fluorescent substrates and protocols employed to determine if NCE inhibit cytochrome P450. Table 5 illustrates data generated on a selection of reference compounds in our lab using recombinant proteins. The final testing concentration in these assays is 1 μM , using the substrates BFC (CYP3A4) or AMMC (CYP2D6). Reference compounds are included every time we run one of these assays. In addition, inhibition also suggests, at a very early point in time, the involvement of a specific cytochrome P450 in the metabolism of a new chemical entity, information that is valuable to pre-clinical investigators.

High-throughput microsomal metabolic stability assays and, in particular, the recombinant P450 inhibition assays are sensitive to the presence of organic solvents (e.g., DMSO, acetonitrile, methanol) in which the NCE are dissolved (90,101–103). DMSO appears to be a universal solvent used in

Table 5 Inhibition of Cytochrome P450 Enzymes by Reference Compounds

CYP3A4			CYP2D6		
Compound	Mean	SEM	Compound	Mean	SEM
Verapamil	31	0.7	Thioridazine	26	0.4
Terfenadine	35	1.2	Chlorpromazine	29	0.5
Cyclosporin A	52	1.4	Promethazine	47	0.9
Astemizole	62	1.3	Terfenadine	56	1.2
Buspirone	91	1.0	Propranolol	74	0.8
Triazolam	94	1.3	Timolol	80	0.7

Results are expressed as percent of activity in solvent controls.

Table 6 The Biopharmaceutics Classification System (BCS)

BCS Class	Solubility	Permeability
I	High	High
II	Low	High
III	High	Low
IV	Low	Low

pharmacological profiling, but it is very detrimental to the activity of many recombinant P450 enzymes. However, all of these common solvents adversely affect activity to some degree and the total concentration of all organic solvents in microsomal incubations should be minimized (e.g., < 0.5%).

Recently, drug metabolism scientists employed in the pharmaceutical industry have gone on record concerning their view of high-throughput pharmaceutical profiling and its place between discovery and pre-clinical assessments of pharmacokinetics and safety (104,105). Furthermore, examples of the entire process, from initial synthesis of NCE through pre-clinical evaluation, have been published (106,107).

Ideally, medicines intended for oral administration should be reasonably soluble, readily absorbed and relatively metabolically stable in order to reach their target at a sufficient concentration. Optimization of oral delivery requires optimization of the pharmaceutical properties discussed here. In fact, a new mechanistic standard for evaluating bioavailability/bioequivalence (108) is based on a biopharmaceutics classification system (BCS). The criteria include the determination of three factors. The first is dissolution number, which is related to aqueous solubility at pH 6.8 and determined by

mean intestinal residence time/drug dissolution time

The second factor is dose number, calculated as
intestinal [drug]/aqueous solubility.

The third factor is absorption number, as follows:

intestinal permeability $\times$ mean intestinal residence time

This strategy facilitates the construction of four possible classes of medicinal compounds (Table 6) based on specific data, some of which can be obtained in vitro before pre-clinical studies begin.

3.4. Cellular Toxicity

As mentioned elsewhere in this text ([Chapter 10](#)), the assessment of drug safety in vivo is prescribed by federal regulations. This process normally follows elaborate, standardized procedures, but there has been a consistent

effort to develop *in vitro* models that are capable of generating data on cellular toxicity before lead candidates are subjected to animal studies. In this effort, QSAR methods have shown some success in relating one indicator of acute toxicity, LD₅₀, to certain physicochemical properties, particularly lipophilicity. However, structure–activity screens that incorporate toxicologic end-points add significant value to lead compound selection and improve development speed. Cytotoxicity *in vitro* is poorly correlated with LD₅₀, but good correlations have been obtained between toxicity *in vitro* and *in vivo*, using systems in which the toxic endpoint reflects the probable mechanisms of acute toxicity (109). Generally, there is agreement that *in vitro* data can eventually make a significant contribution to, and perhaps improve, the determination of human risk. However, remaining objections relate to the extent to which *in vitro* toxicity data should be used to judge potential human toxicity. Clearly, it is important to use batteries of tests capable of evaluating a variety of potential toxic endpoints (110).

In vitro methods may be of doubtful value for general purpose, broad-spectrum toxicity screening of single chemical entities or for priority selection of unrelated chemicals. However, they can be of value for priority selection of homologous series with a known, specific effect (111). However, without other information about the compounds to be tested and compared (e.g., physicochemical properties), interpretation of test results and subsequent comparisons becomes difficult (e.g., comparing lipophilicity rather than toxicity). Under these circumstances, *in vitro* data may not contribute to meaningful risk–benefit assessment and decisions required for medicines (112).

Despite some reluctance to incorporate *in vitro* determinations of cellular toxicity into established drug safety programs, a strategy that incorporates toxicology early in the selection of a lead compound could help reduce risk and prove cost effective. Scientific value may be gained through toxicity studies that identify the mechanism of toxicity. A shift in emphasis is occurring for toxicity testing, with many companies beginning to move investigative toxicity screening from pre-clinical development to drug discovery. Towards this end, the use of exploratory or non-routine studies of the potential of mechanisms of toxicity is becoming more widely adopted in discovery. In the screening mode, *in vitro* assays of cellular injury can be employed to screen several new compounds belonging to similar therapeutic classes in order to rank order the potential for known toxic effects. The results are frequently used to identify and eliminate toxic liabilities much earlier than in the past (113).

The development of *in vitro* models to evaluate the toxicity of xenobiotics in drug discovery has become increasingly important. *In vitro* systems enhance our understanding of the mechanisms of drug-induced toxicity. The use of reference compounds that are known to be toxic and some that have shown to be non-toxic is very important in this process.

As described for the other *in vitro* assays described in this chapter, known toxicants can be compared with the toxicity profile of the unknown agents. By using a battery of cytotoxic endpoints and measurements of cellular function, general cytotoxic characteristics of the compounds can be determined. Therefore, during the past decade, the use of *in vitro* methods/models has expanded greatly in an attempt to reduce the time required by traditional animal testing models (114).

One strategy that has been widely adopted in this regard is the use of biomarkers in toxicity screening. Biomarkers of exposure (e.g., GSH depletion) or effect (e.g., enzyme inhibition/induction) may be used to rank NCE (115). Some of the new technologies used to assess cellular responses have been derived from the human genome project. The most promising technologies with respect to understanding toxic mechanisms include large-scale analysis of gene expression using microarrays (gene chips) and two-dimensional electrophoresis combined with mass spectrometry for analysis of cellular proteins (the proteome). Existing methods for genomic analysis include RT-PCR and branched DNA assays that amplify the target and the signal, respectively. Homogenous, time-resolved fluorescence and fluorescence polarization have recently adapted to measure nucleic acids, but they require further optimization and validation. Large-scale transcript imaging makes it possible to monitor changes in the expression of hundreds or thousands of genes in parallel, using hybridization to gene microarrays or sequencing approaches. However, many toxic responses do not involve changes in mRNA transcription. Analysis of cellular proteins by two-dimensional electrophoresis is not a new technique, but advances in sample preparation and detection have enhanced this technique (116). At this point of time, the primary difficulties associated with the application of these techniques to drug discovery are the vast amount of information generated by a single experiment and the interpretation of the results in the absence of information on the biological and pharmacological significance of observed changes.

Therefore, attempts are underway to use simpler systems to detect biomarkers of exposure and effect as indicators of perturbations in normal cellular physiology. The underlying assumption is that these perturbations could lead to toxic events. In many cases, hepatocytes and hepatoma cell lines (e.g., HepG2 cells) may be used to test NCE for these effects. For this purpose, non-liver-specific end-points for toxicity are used, including plasma membrane integrity (dye uptake, intracellular enzyme or ion leakage), lysosomal integrity, mitochondrial activity, and metabolic competence (protein and DNA synthesis, GSH content, lipid peroxidation) (104,117). One example of this technique is the application of fluorescence microscopy to evaluate the integrity of electron transport pathways via rhodamine reduction (118). However, the routine application of these assays in drug discovery is in its infancy and a great deal of work remains before the value of these determinations can be measured.

4. CONCLUSION

This chapter has been written while pharmaceutical profiling of NCE is in its infancy and when there are still debates about the value of this strategy. Published materials on the process are relatively scarce and the proof-of-principle for pharmaceutical profiling is still being tested. However, the most current research reports on this subject include Kerns and Di (119) and Eddershaw et al. (120). Several generalizations may be made at this time. First, many of the procedures described here are not necessarily applicable across diverse molecular structures or therapeutic areas (e.g., permeability). Instead, these assays appear to function most reliably when homologous series of NCE are tested and compared. Many of the models mentioned here have been validated with small sets of existing medicines and the generalization of these results to new medicinal compounds arising from combinatorial synthesis may not be a valid procedure. Finally, all of these procedures require ongoing validation with the inclusion of a set of suitable reference compounds each time an experiment is conducted.

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Toxicity Evaluations: ICH Guidelines and Current Practice

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1. CHAPTER OVERVIEW

This chapter is provided to address the conduct of non-clinical* toxicity studies in the drug development process and specifically the role of the International Conference on Harmonization (ICH) of the Technical Requirements for the Registration of Pharmaceuticals for Human Use guidances in current toxicology practices. This chapter should be useful to the beginning toxicologist as well as persons in the pharmaceutical industry with no formal training in toxicology, but whose roles may require them to be involved in the design and conduct of the studies. The seasoned toxicologist also might find this chapter to be a good review of current practices. My hope is that this chapter will be a springboard into knowledge and as such, I have provided numerous Internet addresses at which to find additional information.

The chapter is divided into four major sections. In the first section, an introduction to the ICH process is provided. It is important as one moves into the actual ICH topics to have a basic understanding of the parties to

* As these toxicity studies are conducted prior to the initiation of clinical trials, the word “pre-clinical” is often used. However, as toxicity studies are also conducted during the course of clinical trials, it is perhaps more correct to refer to these studies as “non-clinical”.

ICH and the process by which ICH operates to propose and approve guidances for the pharmaceutical industry.

The second section is a presentation of the non-clinical toxicity guidances in the overall safety designation of ICH topics. Among the topics included in this particular section are ICH guidance for single and repeated-dose toxicity studies, reproductive toxicity studies, genotoxicity and carcinogenicity studies, and the safety assessment of biotechnology-derived pharmaceuticals.

The third section provides a short presentation of the non-clinical safety topics that fall outside of the traditional toxicology realm. These non-clinical ICH safety guidances are in the area of pharmacokinetics (PK)/toxicokinetics (TK) and evaluation of systemic exposures in non-clinical studies and in the area of safety pharmacology, an area of intense recent interest.

The fourth and final section is a presentation of case studies involving a series of non-clinical toxicology packages of studies that were submitted to support the marketing approval of specific drugs. These case studies of CelebrexTM, Herceptin[®], Rituxan[®], and Remicade[®], are each illustrative of the unique nature of the different drug development programs. It is my hope that the case studies, together with the ICH guidances, provide a constructive starting point for the design of the package of non-clinical toxicity studies that may be required for a new drug.

2. INTRODUCTION

The ICH is a regulatory and scientific undertaking initiated with the goal of improving the drug development process in the three major regions, Europe, Japan, and the United States, through harmonization of the regulatory guidances among these regions. Established in 1990, ICH is an ongoing joint project between governmental regulatory authorities and pharmaceutical industry experts from each of the three major regions. These six parties to ICH meet with the goal of reaching scientific consensus on various regulatory issues, thereby standardizing the regulatory guidances globally. This standardization of regulatory guidances should significantly reduce the duplication of development activities that might occur when a company desires to obtain worldwide marketing approval. The positive influence of the ICH process is readily apparent in the improved relationship among government regulators and the pharmaceutical industry. The industry is now more able to implement strategies for drug development that allow registration in multiple regions. Much of the credit for the success of ICH has been the end result of the commitment of the regulatory authorities to implement the tripartite harmonized recommendations and guidelines in each of three regions.

2.1. ICH Topics

The ICH has divided the topics into four major categories designated Q, S, E, and M. These categories correspond to the overall areas for drug development and regulatory approval as illustrated in Table 1.

In this chapter, the focus of the presentation will be on safety topics addressed by ICH. For guidances in other areas, the reader is referred to the ICH website (see [Appendix](#)). As will be seen, toxicology study guidelines have been the primary area addressed in the realm of the safety topics. Before proceeding to the discussion of the safety topics, however, it may be beneficial to first review the ICH process and parties involved to understand the basis and context of the current regulations.

2.2. Parties to ICH

The ICH is composed of six representative parties from three regions; regulatory representatives from Europe, Japan, and the United States and pharmaceutical industry representatives from these same three regions. Each of these parties is described briefly below.

2.2.1. European Commission (EC)–European Union (EU)

The EC represents the 15 members of the EU. The EC is working, through harmonization of technical requirements and procedures, to achieve a single market in pharmaceuticals that would allow free movement of products throughout the EU. The European Agency for the Evaluation of Medicinal Products (EMA) was established by the EC in 1993 and is headquartered in London. The EMA is responsible for providing advice and guidance on research and development programs and evaluating pharmaceutical products for human use, and the EC subsequently authorizes the marketing of products on the basis of the EMA's opinion. Technical and scientific support for ICH activities is provided by the EC via the Committee for Proprietary Medicinal Products (CPMP) of the EMA.

Table 1 Four Categories of ICH Topics

Letter designation	Category	Topic
Q	Quality	Guidances in the area of chemistry, manufacturing, and control (CMC)
S	Safety	Guidances in the area of non-clinical toxicology, pharmacology, and PK/TK
E	Efficacy	Guidances in the area of clinical studies of safety and/or efficacy
M	Multidisciplinary	Guidances that cross-categorical lines

2.2.2. European Federation of Pharmaceutical Industries' Associations (EFPIA)

The EFPIA is situated in Brussels and is made up of member associations in 16 countries in Western Europe. These member associations ensure that the EFPIA's views of proposed ICH guidelines are representative of the pharmaceutical industry in the EU. Companies in membership of EFPIA are manufacturers of prescription medicines and include all of Europe's major research-based pharmaceutical companies.

2.2.3. Japan—Ministry of Health, Labour, and Welfare (MHLW)

The MHLW Ministry was formed in 2001 from the Japanese Ministry of Health and Welfare and the Japanese Ministry of Labour. The MHLW has, as one of its responsibilities, the protection and promotion of the health and welfare of the Japanese people. The MHLW is responsible for a wide range of administrative activities encompassing the approval of drugs as safe and effective.

2.2.4. Japan Pharmaceutical Manufacturers Association (JPMA)

The JPMA represents 90 member companies. Membership includes all of the major research-based pharmaceutical manufacturers in Japan. Among the objectives of JPMA is the development of a competitive pharmaceutical industry with a greater public awareness and understanding of issues in the development of new pharmaceuticals. The JPMA promotes and encourages the adoption of international standards by its member companies.

2.2.5. United States—Food and Drug Administration (FDA)

The FDA consists of administrative, scientific, and regulatory staff organized under the Office of the Commissioner and has several centers with responsibility for the various products that are regulated. Technical advice and experts for ICH work are drawn from the Center for Drug Evaluation and Research (CDER) and the Center for Biologics Evaluation and Research (CBER).

2.2.6. Pharmaceutical Research and Manufacturers of America (PhRMA)

The PhRMA represents the research-based industry in the USA. The association has 67 companies in membership that are involved in the discovery, development, and manufacture of prescription medicines. There are also 24 research affiliates that conduct biological research related to the development of drugs and vaccines. The PhRMA, which was previously known

as the US Pharmaceutical Manufacturers Association (PMA), coordinates its technical input to ICH through its Scientific and Regulatory Section. Special committees of experts from PhRMA companies have been created to deal with ICH topics.

2.2.7. Additional Participants

Observers are the World Health Organization (WHO), the European Free Trade Area (EFTA) represented at ICH by Switzerland, and Canada represented at ICH by the Therapeutic Products Directorate (TPD), Health Canada. Each of the Observer parties has a seat on the ICH Steering Committee.

In addition, the International Federation of Pharmaceutical Manufacturers Association (IFPMA) is a federation of member associations representing the research-based pharmaceutical industry and other manufacturers of prescription medicines in 56 countries throughout the world. The IFPMA has been closely associated with ICH since its inception to ensure contact with the research-based industry outside the ICH regions. The IFPMA has two seats on the ICH Steering Committee and runs the ICH Secretariat.

2.3. Steps in the Process of Harmonization in the ICH Process

The approval process of ICH guidelines is a 5-step process. The status of each proposed or implemented guidance is provided on the ICH web site.

Step 1: Consensus building

An initial draft of a guideline (or recommendation) is prepared by the ICH Rapporteur and the ICH Expert Working Group (EWG). This guideline is prepared in consultation with experts designated to the EWG. When consensus on the guideline is reached, the final revised consensus guideline is submitted by the EWG to the ICH Steering Committee for adoption and the guidance moves to the next step in the process.

Step 2: Start of Regulatory Action

When the Steering Committee agrees that the proposed guideline contains sufficient scientific consensus on the technical issues laid out in the guideline, the draft guideline is deemed ready to go to regulatory consultation stage. The six parties to the ICH confirm, with signatures, that the ICH Scientific Committee agrees with the ICH EWG proposed guideline and the guidance moves to the next step.

Step 3: Regulatory Consultation

At this stage, the guideline is circulated by the three regions for regulatory consultation; in the EU it is published as a draft CPMP Guideline, in Japan it is issued by the MHLW and in the United States it is published as a draft guidance in the Federal Register. With this circulation comes the

opportunity for regulatory authorities and industry persons from non-ICH regions to comment on the draft document. The three regulatory parties review these comments with the goal of reaching a single, harmonized wording of the guideline. The final revised guideline is approved by the regulatory parties of the three regions.

Step 4: Adoption of a Tripartite Harmonized Text

Since the guideline may have been revised from that proposed by the ICH Steering Group, at Step 4 the guideline is returned to ICH and reviewed by both industry and regulatory experts to ensure that the proposals in the guideline remain acceptable subsequent to the consultation edits. If both regulatory and industry delegates are in agreement with the guideline, the text of the guideline is adopted and the guideline signed by the three regulatory parties to ICH; at this point the guideline is recommended for adoption by the regulatory bodies in the three regions.

Step 5: Implementation

The Tripartite Harmonized Guideline is implemented by the regulatory bodies. In the European Union, the Guideline is published by the European Commission in Volume III of the Rules Governing Medicinal Products in the European Union. In Japan, the Pharmaceutical and Medical Safety Bureau (PMSB) is responsible for the promulgation of the Guideline. In the United States, the Guideline is published by FDA in the Federal Register.

3. ICH NON-CLINICAL (PRECLINICAL) TOXICITY GUIDELINES

The non-clinical toxicity testing of a new drug (including biotechnological products) is a fairly well-defined process in terms of the five general areas of testing, although the actual studies and protocol elements of the studies within each of these general areas may vary depending on the class of drug and intended clinical program. In this section, ICH activity is discussed and additional comments are provided regarding study design in each of the following areas of toxicity testing:

- Single-dose toxicity
- Repeat-dose toxicity
- Reproductive toxicity
- Genetic toxicity
- Carcinogenicity

A tabular listing of the ICH guidances in the “Safety” and “Multidisciplinary” categories that are specifically toxicity testing guidances is provided in [Table 2](#). All of these guidances have reached the Step 4 adoption of the guidance text and have been implemented by the regulatory bodies from each of the three regions (Step 5).

Table 2 ICH Non-clinical Toxicity Testing Guidelines

Topic designation	Title
S1	CARCINOGENICITY STUDIES
S1A	Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals
S1B	Testing for Carcinogenicity in Pharmaceuticals
S1C	Dose Selection for Carcinogenicity Studies of Pharmaceuticals
S2	GENOTOXICITY STUDIES
S2A	Genotoxicity: Specific Aspects of Regulatory Tests
S2B	Genotoxicity: Standard Battery Tests
S4	TOXICITY TESTING
S4	Single Dose Toxicity Tests
S4A	Duration of Chronic Toxicity Testing in Animals (Rodent and Non-Rodent)
S5	REPRODUCTIVE TOXICOLOGY
S5A	Detection of Toxicity to Reproduction for Medicinal Products
S5B	Reproductive Toxicology: Male Fertility Studies
S6	BIOTECHNOLOGICAL PRODUCTS
S6	Safety Studies for Biotechnological Products
M	MULTIDISCIPLINARY
M3	Timing of Pre-clinical Studies in Relation to Clinical Trials

3.1. Single-Dose Toxicity Guidance (ICH Topic S4)

One of the first guidances implemented by ICH was the guidance regarding single-dose toxicity. The main intent of this guidance was to remove the classical acute lethality dose determination (LD_{50}) from acute toxicity testing protocols, thereby altering the objective of these studies from one of determining the dose that leads to death (and perhaps an inordinate amount of suffering to the animal) to one of determining the maximum tolerated dose (MTD). This guidance was agreed upon prior to the first ICH meeting in 1991 and was published in the Proceedings of the First International Conference on Harmonization. In the United States, the guidance was published in the Federal Register with FDA revision (1). This FDA revision modified the ICH guidance to include wording that would allow the use of single-dose toxicity studies to support single-dose clinical trials in humans; for example “in the screening of multiple analogs to aid in the selection of a lead compound for clinical development.” This modification is consistent with the ICH position on acute toxicity testing, but should be noted to be an FDA-specific modification of the ICH guidance.

The ICH guidance provided some specific protocol elements to be addressed in the design of single-dose toxicity studies. The range of doses should include those doses that cause no adverse effect to those that cause life-threatening (but not death as an endpoint) toxicities. The drug should be administered by two routes, the intended clinical route and the intravenous (i.v.) route, and should be administered to at least two mammalian species, including a non-rodent species. In contrast to previous studies that required large numbers of animals to calculate lethality parameters, the ICH guidance calls for a limited number of animals, for example 3–5 rodents per sex per group and even smaller numbers of non-rodents (not actually specified as to number). In the investigations in non-rodents, acute dose range-finding (RF) studies may be acceptable to provide the requisite acute toxicity data. The animals should be monitored for 14 days subsequent to dosing at which time a gross necropsy should be conducted.

The FDA guidance adds additional study elements for those special instances in which the data will be used to provide support for single-dose clinical trials (1). The guidance calls for the study to include pharmacokinetics and assessment of dose–response relationships and for more detailed toxicity assessments to include clinical pathology and histopathology evaluations at an early time (at which toxicity might be expected to be greatest) and later at termination (14 days) to evaluate recovery.

3.2. Repeat-Dose Toxicity Guidances

Repeat-dose toxicity studies in laboratory animals form the crux of the studies characterizing the potential toxicities of the compound. The toxicologist desires to identify the target organs for toxicity in the animal and potential surrogate markers in man, define the dose-response relationships for any observed toxicities including the threshold dose for observing toxicities, determine systemic exposures resulting in toxicities for extrapolation to man, and determine the potential for reversibility of observed toxicities.

Two questions come immediately to mind when considering repeat-dose toxicity studies in the overall development plan; “How long should each particular toxicity study be?” and “When do the studies need to be conducted to support clinical trials?” Both of these questions have been addressed in the ICH guidances.

3.2.1. Duration of Toxicity Testing (ICH Topic S4A)

Prior to the ICH guidance regarding the length of a chronic toxicity study, there was general consensus across the three regions that a 6-month duration was sufficient for a chronic toxicity study in rodents and, hence, the ICH S4A guidance regarding the study length in rodents was readily harmonized at 6 months (2). However, the length of the chronic repeated-dose toxicity study in non-rodents was different in the three regions. In the United States, the

FDA generally held that the repeated-dose toxicity study in non-rodents be 12 months in duration to be considered chronic, whereas in Japan and Europe, a repeat-dose toxicity of 6 months duration was considered sufficient to support long-term clinical trials. Consequently, both 6- and 12-month studies were often being performed in non-rodents; an action that might be considered to be a redundant use of animals. Following a review of 6- and 12-month data by regulatory reviewers from all three regions, ICH proposed and reached agreement that a study duration of 9 months in non-rodents would be considered an acceptable duration for a chronic toxicity study. This harmonization is an excellent example of the ICH process meeting its goal to eliminate duplication of testing.

3.2.2 Timing of Conducting Preclinical Toxicity Studies (ICH Topic M3)

The timing of when to conduct the repeat-dose toxicity studies to support clinical trials is of utmost importance. The timeline for initiation of each phase of clinical trial (Phase I, II, and III) is the critical path in the later stage development of the compound. To support each of these stages of clinical trials, a combination of animal toxicity data and previous clinical trial data is used to progress to the subsequent clinical trial. ICH Guidance M3 is a multidisciplinary guidance that defines at a particular stage of clinical development the realm of preclinical safety studies generally required to proceed with a particular clinical trial (3).

The durations of the repeat-dose toxicity studies in laboratory animals need to be evaluated in light of the duration, therapeutic indication, and scale of the proposed clinical trials. In principle, the duration should be equal to, or exceed, the duration of the clinical trial up to the maximum recommended duration of the repeat-dose toxicity studies; the studies should be conducted in two species, including a non-rodent species. The proposed durations of repeated-dose toxicity studies are presented in [Tables 3](#) and [4](#).

There are several points in these tables regarding the duration of repeated-dose toxicity testing that bear mentioning. As discussed in section on Single-Dose Toxicity Guidance, the United States will allow single-dose toxicity studies to support single-dose Phase I trials provided that these single-dose studies include clinical pathology determinations and histopathological assessment at early and later time points. However, this is not universally accepted. For clinical trials of longer duration, the guidance also provides for the possible conduct of a 6-month repeat-dose toxicity study in non-rodents (Table 3), a study that, as discussed on p. 12, is not required under the ICH guidance regarding length of chronic preclinical toxicity studies. This type of 6-month repeat-dose study would only be required if the data were needed to support a clinical trial of longer than 3 months, and if 9-month repeat dose data in a non-rodent species were not available. The likeliest scenario if time were absolutely crucial to the initiation of a clinical

Table 3 Duration of Repeated-Dose Toxicity Studies to Support Phase I and II Trials in EU and Phase I, II, and III Trials in the United States and Japan^a

Duration of clinical trials	Minimum duration of repeated-dose toxicity studies	
	Rodents	Non-rodents
Single dose	2 weeks ^b	2 weeks
Up to 2 weeks	2 weeks	2 weeks
Up to 1 month	1 month	1 month
Up to 3 months	3 months	3 months
Up to 6 months	6 months	6 months ^c
>6 months	6 months	Chronic (9 months) ^c

^aIn Japan, if there are no Phase II clinical trials of equivalent duration to the planned Phase III trials, conduct of longer duration toxicity studies is recommended as given in Table 4.

^bIn the United States, as an alternative to 2-week studies, single-dose toxicity studies with extended examinations can support single-dose human trials.

^cData from 6 months of administration in non-rodents should be available before the initiation of clinical trials longer than 3 months. Alternatively, if applicable, data from a 9-month non-rodent study should be available before the treatment duration exceeds that which is supported by the available toxicity studies.

6-month trial would be to conduct a preclinical 9-month study with an interim necropsy at 6 months. An important regional difference is illustrated in Table 4, where the length of repeated-dose toxicity studies to support Phase III clinical trials in Europe is longer than in Japan and the United States.

The enrollment of men and women in clinical trials and their reproductive capability, or impairment of such function, is of considerable importance and is addressed in this ICH Guidance (please refer to [Section 3.4](#) for the ICH Guidances regarding specific reproductive toxicity studies). The

Table 4 Duration of Repeated-Dose Toxicity Studies to Support Phase III Trials in the EU and Marketing in all Regions^a

Duration of clinical trials	Minimum duration of repeated-dose toxicity studies	
	Rodents	Non-rodents
Up to 2 weeks	1 month	1 month
Up to 1 month	3 months	3 months
Up to 3 months	6 months	3 months
>3 months	6 months	9 months

^aThis table reflects the Marketing recommendations in the three regions except that a chronic non-rodent study is recommended for clinical use >1 month.

Table 5 Protocol Elements of Subchronic Toxicity Studies (2–13 Weeks Duration)

Protocol elements	USA	Europe	Japan
Species	One rodent and one non-rodent species; rats and dogs preferred	One rodent and one non-rodent. One species may be acceptable if it is the only relevant species	One rodent and one non-rodent (rabbit cannot be considered as non-rodent species)
Age at study start	Rats 6 weeks. Dogs 4–6 months	Not defined	Not defined
Number and size of groups	At least three treated and one control. For rodents, 10/sex/group, individually housed. For non-rodents, at least 4/sex/group (2/sex/group for a pilot study)	Three treated, one control. Group sizes not specified, depends on design including interim and recovery sacrifices	At least three plus vehicle control, untreated control group may be necessary. For rodents, 10/sex/group, individually housed. For non-rodents, 3/sex/group
Recovery period and TK evaluation	Recovery groups recommended, systemic exposure of oral dose should be ensured	Recovery groups recommended, TK are essential to include	Recovery groups and TK are recommended
In-life observations	Observations twice daily, ^a body weight, and food consumption weekly, water consumption only if administered in drinking water. Ophthalmology at baseline and termination. ECG not addressed	Observations daily, body weight, food consumption, ophthalmology in rodents and non-rodents required but frequency not defined. ECG not addressed	Observations daily, body weight and food consumption weekly, ophthalmology in all animals at least once during study, ECG in non-rodents as appropriate
Clinical pathology	Hematology, clinical chemistry, urinalysis in 10 rodents/sex/group and in all non-rodents at	Hematology, clinical chemistry, urinalysis required. Important to include	Hematology and clinical chemistry in all animals at necropsy, urinalysis once during study.

(Continued)

Table 5 Protocol Elements of Subchronic Toxicity Studies (2–13 Weeks Duration)
(Continued)

Protocol elements	USA	Europe	Japan
	predose and termination	immunotoxicity evaluation in at least one repeated-dose rodent study	Rather than allowing death, euthanize moribund animals to collect clinical pathology data
Histopath	For rats, full histopath evaluation on all high dose and control and moribund/dead animals, target tissues in mid and low dose groups and recovery groups. Full histopath on all non-rodent species all dose groups	For rats, full histopath evaluation on all high dose and control and moribund/dead animals, target tissues in mid and low dose groups and recovery groups. Full histopath on all non-rodent species all dose groups	For rats, full histopath evaluation on all high dose and control animals. Full histopath on all non-rodent species all dose groups

^aThis likely refers to mortality checks and not detailed and recorded clinical signs.

ICH M3 guidance draws special attention to the enrollment of women of child-bearing potential and maintains some regional differences in the timing of reproductive toxicity studies to support clinical trials involving this population. The assessment of embryo-fetal development should be completed prior to the enrollment of women of child-bearing potential in both Europe and Japan. Japan goes further by suggesting that female fertility studies as well be conducted prior to enrollment of women. In Europe, female fertility studies should be completed prior to Phase III trials.

In the United States, the inclusion of women of child-bearing potential has been an issue for some time. Historically, women were excluded from early clinical trials in the United States due to concern over birth defects in children of treated mothers. The 1977 FDA Guideline “General Considerations for the Clinical Evaluation of Drugs” (www.fda.gov/cder/guidance/old034fn.pdf) states that “In most cases, women of

Table 6 Protocol Elements of Chronic Toxicity Studies (6 Months in Rodents and 9 Months in Non-rodents)

Protocol elements	USA	Europe	Japan
Species	Same as subchronic	Same as subchronic	Same as subchronic
Age at study start	Same as subchronic	Same as subchronic	Same as subchronic
Number and size of groups	Same as subchronic except for rodents, 20/sex/group, increase size by 10/sex/group for each interim necropsy	Same as subchronic	Same as subchronic
Recovery period and TK	Recovery groups not routinely used, toxicokinetics useful to include	Same as subchronic	Same as subchronic
In-life observations	Same as subchronic; body weight and food consumption measured weekly to 13 weeks and monthly thereafter	Same as subchronic	Same as subchronic; body weight and food consumption measured weekly to 3 months and every 4 weeks thereafter
Clinical pathology	Hematology, clinical chemistry, urinalysis in 10 rodents/sex/group at predose, Days 30 and 60, and termination and in all non-rodents at pre-dose and at 3-month intervals thereafter	Hematology, clinical chemistry, urinalysis required	Same as subchronic
Histopath	Same as subchronic	Same as subchronic	Full histopath evaluation on all animals, rodents and non-rodents

childbearing . . . should be excluded from Phase I.” However, more recently, the FDA has initiated regulatory reforms to address the perceived barrier to the enrollment of women in clinical trials in 1993 by emphasizing the critical importance of including women in all phases of clinical trials (<http://www.fda.gov/cder/guidance/women.pdf>), and in 1998 by amending its regulations to require effectiveness and safety data across demographic subgroups including women (4), and imposing the possibility of a penalty of a clinical hold on studies under an IND if women with reproductive potential are excluded from participation in a study only because of the risk or potential risk of reproductive or developmental toxicity from use of the drug (5). Under the ICH M3 guidance, women of child-bearing potential in the United States may be included in early, carefully monitored trials without reproduction toxicity studies in animals. Nonetheless, due to United States guidances stressing inclusion of women and the requirement for teratogenicity studies in animals prior to Phase I in Japan and Europe, it is becoming common for pharmaceutical companies to include reproductive studies, notably embryo-fetal development studies, at an early stage of the drug development process in the United States. Under ICH M3 guidance, the recommendation in the United States is that the assessment of embryo–fetal development and female fertility should be completed prior to Phase III.

The ICH guidance is more straightforward with regard to the timing of reproduction toxicity studies and the enrollment of men in clinical studies. The consensus across the three regions is that men may be included in Phase I and II trials since the male reproductive organs are part of the gross anatomic and histopathological tissues for examination in repeat-dose toxicity studies. The male fertility study should be completed prior to initiation of Phase III trials enrolling men. Likewise, with regards to the timing of the pre- and post-natal development study in animals, there is consensus across the three regions that the study should be submitted for marketing approval and hence, can be conducted late in the drug development program.

The ICH M3 guidance provides recommendations for the timing for conducting local tolerance studies, genotoxicity studies, and carcinogenicity studies. Local tolerance should be evaluated using the relevant clinical route. Since repeat-dose toxicity studies generally involve the clinical route of administration, these studies can evaluate local tolerance as well by including, for example, histopathological evaluation of administration site. The ICH guidance suggests that the genotoxicity package of studies be at least partially completed by the time of first human exposure, specifically, in vitro assays for mutagenicity and chromosomal damage. The entire battery of testing recommended in ICH guidance S2A and S2B (see [Section 3.5](#)) should be completed prior to Phase II clinical studies. Regarding carcinogenicity testing, these studies are generally not required to support clinical trials;

these studies, if required, may be conducted prior to marketing approval or as a post-marketing commitment (see [Section 3.6](#)).

In terms of clinical trials in pediatric populations, the ICH M3 guidance states that human adult clinical trial data will be most relevant to the conduct of pediatric trials. Juvenile animal repeat-dose toxicity studies should be considered when previous animal and human data might be considered insufficient. Lastly, the guidance calls for all reproduction toxicity testing (p. 12), all genotoxicity testing (p. 14), and the appropriate repeated-dose toxicity studies be completed prior to initiation of pediatric clinical trials.

The ICH M3 guidance also mentions the timing of safety pharmacology and PK/TK studies; these specific ICH guidances are addressed in [Section 4](#). Safety pharmacology studies should be evaluated prior to any human exposures. These evaluations may be conducted as stand-alone studies and/or in combination with a standard toxicity study. Pharmacokinetic data in animals should be available by the time the first clinical trial in humans is conducted. In order to compare the absorption, distribution, metabolism, excretion (ADME) characteristics of the drug in humans, animal studies should be completed by the time that Phase I clinical trials are completed.

3.3. Additional Guidance Information and Protocol Elements in Repeated-Dose Toxicity Studies

All three regions (USA, Europe, and Japan) provide additional guidance with respect to protocol elements to be included in repeated-dose toxicity studies (6–8). In the United States, recommendations are provided in the Redbook, a Center for Food Safety and Applied Nutrition set of guidances for the safety assessment of food additives. Although not directly written to address drugs and biologics, the guidances are often referred to by default. The regional protocol elements are provided in [Tables 5](#) and [6](#), with some further discussion and recommendations for the reader. The actual listing of clinical pathology parameters and of the tissues to be collected for histopathological examination are not provided in these tables, but can be found in the guidances as well as in the literature (9).

The elements of repeat-dose subchronic toxicity testing are listed in [Table 5](#). The recommended species for these tests are generally rats and dogs. Repeat-dose studies in mice are generally conducted with the primary objective (notably in the 13-week study) of establishing dose levels for a carcinogenicity bioassay and not with the goal of defining no adverse effect levels (NOAEL) or target organs for toxicity. An important consideration for subchronic repeat-dose toxicity studies in rodents is the emphasis in Europe on including an immunotoxicity component in at least one 28-day repeated-dose study in rodents; bone marrow cellularity, lymphocyte subsets, and natural killer cell activity or the primary antibody response

Table 7 Reproductive Toxicity Testing—3-Study Design

Number designation	Study title	Recommended species
4.1.1	Fertility and early embryonic development	Rats
4.1.2	Pre- and post-natal development	Rats and rabbits
4.1.3	Embryo–fetal development	Rats

to a T-cell-dependent antigen are recommended (7). The FDA is also moving rapidly to address the importance of immunotoxicological evaluation of new drugs (10). Recovery groups of animals are usually included in subchronic toxicity studies, especially those studies of 4 weeks in duration; the recovery period is usually 2–4 weeks. The guidances provide general recommendations for clinical pathology evaluations in rodent and non-rodent studies. In a 13-week study, clinical pathology determinations might also be recommended at a 1-month interim if the treatment levels are different from those used in a 4-week study.

The protocol elements for chronic toxicity testing are listed in [Table 6](#). Note that the protocol elements of the chronic toxicity studies are similar to those listed for the subchronic toxicity studies. The FDA recommends an increase in rodent group size to 20/sex/group. However, a group size of 15/sex/group should be sufficient to address target organ toxicity. With regard to a recovery period, recovery groups are usually not included in chronic studies since reversibility has usually been demonstrated at this point in the toxicity evaluation; including recovery groups prolongs the study duration at a time when these chronic toxicity studies may lie on the critical path for the development of the drug. Full TK profiles do not generally need to be determined for a chronic study unless the study uses dose levels for which no data exist. To substantiate systemic exposures and provide comparisons to subchronic toxicity studies, a limited blood sample collection for drug concentration analysis of 2–3 time points at 3-month intervals is recommended. Clinical pathology evaluation in rodents should be conducted at the same 3-month intervals as non-rodents. Rodents should be randomly allocated into subgroups of 10/sex/group (in other words, not all of the rodents in each group need to be evaluated) from which clinical pathology parameters are collected. Ophthalmologic evaluation in rodents and non-rodents, and ECG collection in non-rodents should be collected at 3-month intervals; in rodents, again it is feasible to use subgroups of 10/sex/group rather than the full 15/sex/group.

3.4. Reproductive Toxicity Guidelines

One of the first topics addressed by ICH was the area of reproductive toxicity testing. The complexity of the studies and the questions being addressed

Table 8 Standard Battery of Tests for Genotoxicity Assessment Including Specific Test Elements

Core battery testing	ICH Topic S2 Guidance	Additional author comments
Bacterial mutagenicity	<i>Salmonella typhirium</i> strains TA 98, 100, 1535, and TA 1537 or TA 98 or TA 97a. In addition, <i>S. typhirium</i> strain TA 102 or <i>E. coli</i> strains WP2uvrA or WP2PKM101 Highest concentration should show significant cytotoxicity For soluble non-toxic compounds, limit concentration at 5 mg/mL for bacteria and higher of 5 mg/mL or 10 mM for mammalian cells For poorly soluble compounds, use lowest precipitating concentration at high dose up to limit concentration above	Non-audited RF cytotoxicity assay to select dose levels causing decreased viability Two independent assays recommended. In the first, plate incorporation method is suggested. If a negative response is obtained, the liquid preincubation method with metabolic activation is suggested Definite assay should include at least five dose levels tested in triplicate Test substance considered positive if revertant colonies number more than twice the negative control and if increase is dose-dependent
In vitro mammalian mutagenicity	Mouse lymphoma tk test (MLA) recommended; appropriate assays also include HPRT test with CHO-cells, V79-cells, or L5178 cells, GOT-(XPRT) test with AS52 cells, and human lymphoblastoid TK6 test 3–4 hr treatment (MLA); if negative, include a 24 hr treatment in absence of metabolic activation (MLA)	Mouse lymphoma TK assay on L5178Y cell line using microtiter method recommended Non-audited preliminary cytotoxicity assay to select concentrations producing 20–100% survival Two independent mutagenicity assays

(Continued)

Table 8 Standard Battery of Tests for Genotoxicity Assessment Including Specific Test Elements (*Continued*)

Core battery testing	ICH Topic S2 Guidance	Additional author comments
In vitro chromosome aberration	±S9 fraction (MLA)	
	Highest concentration produces at least 80% toxicity	At least four concentrations tested in triplicate
	Include solvent control and positive test compound CHO-WBL cell line or human lymphocytes	Non-audited preliminary cytotoxicity assay to select concentration producing <50% survival
In vivo mutagenicity	Highest concentration produce >50% reduction in cell number for cell lines, or >50% inhibition of mitotic activity in lymphocyte cultures	Two independent clastogenicity assays
		At least three concentrations tested in triplicate
	± S9 fraction	
	3–6 hr exposure	Two harvest times (18 and 42 hr)
	Sampling time of approximately 1.5 normal cell cycles for beginning of treatment	200 metaphases scored/concentration
	Mice or rats acceptable	Mice preferred by Japan
	Chromosome aberration in bone marrow cells or measurement of micronuclei in bone marrow cells both acceptable	Five animals/dose
		3–4 dose levels; top dose = MTD
	Males sufficient, unless there are clear qualitative differences in metabolism	Single dose—oral, i.p. or i.v.
	Suggest TK to evidence systemic exposure	Bone marrow sampling times 24 (all doses) and 48 hr (top dose only) after administration

resulted in numerous and diverse protocols of testing strategies employed across the different countries. The ICH has provided considerable guidance and harmonization of reproductive toxicity testing in ICH Topic S5A and S5B (11,12).

In addition to drug products, reproductive toxicity is a component of testing in the chemical realm as well; for example, pesticides, environmental chemicals, and workplace chemicals. Interestingly, the testing for these chemicals often encompasses multigeneration reproductive toxicity testing, the rationale being that chemical exposures in the workplace and in the environment may occur unexpectedly over ill-defined periods of times. The question may be raised as to why multigenerational studies are not part of the reproductive toxicity testing of drug products. The ICH comments on this and points out that reproductive toxicity testing with medicinal products is much more defined. Hence, the reproductive toxicity testing at specific stages of reproduction is more reflective of humans taking drug products at specified periods and therefore is a better assessment of actual human risk.

As an aside, the FDA has published a draft guidance regarding the integration and interpretation of study results obtained in the reproductive toxicity studies and this is an additional useful resource for the reader (13).

3.4.1. Detection of Toxicity to Reproduction for Medicinal Products (ICH Topic S5A)

ICH S5A provides a three-study combination that should be sufficient for testing of most drug products (see [Table 7](#)). Rats are the primary rodent species, and rabbits the primary non-rodent second species for embryo–fetal effects. The rationale for both is similar and is based on large litter size, predictable gestation period, ease of handling and housing, and most importantly, the large historical background information available.

The study protocol elements for each of these studies are fully described in the ICH guidance document (11,12). A comprehensive summary of the guidance is beyond the scope of this chapter. For the purposes of this chapter, only the major harmonization highlights will be presented.

ICH Study 4.1.1 looks for effects in males and females from before mating to implantation. Importantly, the duration of treatment for males has been shortened significantly from 9–10 to 4 weeks (and subsequently shortened to 2 weeks in ICH S5B). A 1:1 mating ratio is suggested in this guidance, with the sacrifice of males delayed until the outcome of mating is known to allow for re-mating with untreated females if necessary. Females are terminated between gestation days 13–15, in contrast to gestation days 20–21; this time is considered adequate to assess fertility and reproductive indices.

ICH Study 4.1.2 is an evaluation of the pre- and post-natal effects of the drug. Dosing is initiated in the first generation dams (F0) at the implantation stage and continues through to weaning of the first generation (F1),

Table 9 Protocol Elements of Long-Term Carcinogenicity Studies

Protocol elements	USA	Europe	Japan
Species	Two recommended, rats and mice	Rats recommended in absence of evidence for a more appropriate species	Two recommended, rats, mice, or hamsters
Duration	104 weeks	24 months (rats) and 18 months minimum for mice and hamsters	24 months (rats) and 18–24 months for mice and hamsters. Survival should not be less than 50% in low and control groups at termination
Age at study start	6 weeks	As soon as possible after weaning and acclimation	6 weeks
Dose frequency	Daily	Daily	Daily in feed, but oral gavage 5 days/week is acceptable
Number and size of groups	Three treated and one vehicle control. Minimum of 50/sex/group	Three treated and one vehicle control. Minimum of 50/sex/group	Three treated, one vehicle control, one non-treated control. Minimum of 50/sex/group
TK	No	No	No
In-life observations	Observations twice daily, body weight and food consumption weekly to 13 weeks and monthly thereafter	Observations daily, body weight, food consumption required but frequency not defined	Observations daily, body weight and food consumption weekly to 3 months and every 4 weeks thereafter
Clinical pathology	Hematology, clinical chemistry, urinalysis at predose and months 3, 6, 12, 18, and	Hematology, clinical chemistry, urinalysis requested but frequency and animal numbers	Only hematology evaluation is requested, animal number and frequency not defined

(Continued)

Table 9 Protocol Elements of Long-Term Carcinogenicity Studies (*Continued*)

Protocol elements	USA	Europe	Japan
	termination; number of animals not defined	not defined	
Histopath	Histopath evaluation on all high dose and control and moribund/dead animals, target tissues in mid and low dose groups	Histopath evaluation on all high dose and control and moribund/dead animals, target tissues in mid and low dose groups	Histopath evaluation on all high dose and control and moribund/dead animals, target tissues in mid and low dose groups

while observations are continued through to sexual maturity of the F1 generation to allow for the appearance of any delayed effects. ICH S5A recommends that one male and one female from the F1 generation be used for both behavioral/functional testing and testing of reproductive function since such dual use will allow for correlations among the assessments. However, ICH S5A does accept that some laboratories use separate sets of animals and this is accepted in the guidance as being valid as well. One important note in this ICH guidance is the use of culling of the F1 population. This is recognized as a controversial issue among the three regulatory regions, and the issue is still under discussion. Finally, since the study design does not cover exposures of the F1 generation from weaning to maturation, if the intended clinical population is infants and/or juveniles, the potential toxicity of drug products on these age groups should be considered with separate studies unique to the age group in question.

ICH Study 4.1.3, an evaluation of embryo–fetal effects of the drug, is the only guidance in the reproductive toxicity studies that requires two species unless there is strong rationale to conduct such an assessment in a single species. If this embryo–fetal study is conducted in a single study or two study combination of fertility and/or pre- and post-natal development in rodents, an embryo–fetal study must still be performed in the second non-rodent species. The litter size in ICH S5A is standardized at 16–20 litters. The guidance accepts that examination of offspring in the low- and mid-dose groups for visceral and/or skeletal abnormalities may not be necessary if the high dose and control groups show no significant treatment-related differences.

Lastly, the selection of dose levels is imperative as in any testing of toxicity. As such, RF studies are often conducted prior to the definitive studies. While the ICH guidance does not state a requirement for an initial dose RF study, such RF studies are usually included in the conduct of the embryo–fetal development studies (ICH Study 4.1.3); RF studies are not needed for ICH Studies 4.1.1 and 4.1.2 in rats since repeat-dose toxicity data usually exist for this species at the time these studies are to be conducted. The RF embryo–fetal development studies may follow one of three designs: (1) evaluation of maternal toxicity only with animals terminated after final exposure; (2) evaluation of maternal and fetal toxicities where the fetuses are delivered and examined externally (no detailed evaluation of skeletal and visceral abnormalities); and (3) study conducted as if it were the definitive study with full fetal skeletal and visceral examinations. In rats, it is usually sufficient to conduct design 2 as the only RF study. In rabbits, it may be preferable to conduct design 1 first to avoid excessive maternal toxicity and effects on the fetus that may result from an overly excessive maternal toxicity. Alternatively, one may conduct a study along the lines of design 2 with 4–6 treatment dose levels as opposed to the usual three treatment groups. In any instance, even though these studies are RF, they should still be conducted under adherence to GLP guidelines. The norm is also to include collection of blood samples from satellite animals in these RF studies for TK purposes. If TK samples are not collected in the RF studies, they should be collected in the definitive studies to provide an assessment of systemic exposures.

3.4.2. Maintenance of the ICH Guideline on Toxicity to Male Fertility (ICH Topic S5B)

This ICH guidance shortens the duration of exposure of males in Study 4.1.1 (the assessment of drug effect on fertility) from 4 to 2 weeks, provided that there is no indication of male reproductive toxicity in repeat-dose studies of at least 2 weeks in duration.

3.5. Genotoxicity Guidelines

The ICH guidance provides a harmonized battery of tests to be used to investigate the potential genotoxicity of a drug; this battery is detailed in ICH Topic S2B (14). ICH also provides some specific protocol elements and guidance regarding interpretation of test results in ICH Topic S2A (15).

3.5.1. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (ICH Topic S2A)

Whereas ICH S2B provides the overall battery of genotoxicity studies to be conducted for a new drug, ICH S2A provides guidance as to the protocol

Table 10 Additional Preclinical Safety Topics

Topic designation	Title
S3	PHARMACOKINETICS AND TOXICOKINETICS
S3A	Toxicokinetics: Guidance on the Assessment of Systemic Exposure in Toxicity Studies
S3B	Pharmacokinetics: Guidance for Repeated-Dose Tissue Distribution Studies
S7	PHARMACOLOGY STUDIES
S7A	Safety Pharmacology Studies for Human Pharmaceuticals
S7B	Safety Pharmacology Studies for Assessing the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals

elements in each of the different *in vitro* and *in vivo* tests. The core battery of testing recommended by ICH and specific protocol elements are provided in [Table 8](#).

As illustrated in Table 8, ICH S2A outlines the standard base set of strains to be used in the bacterial mutation assay; an important consideration since each of the three global regions had particular preferences to be included in this assay. ICH S2A recommendations for the *in vitro* assays include the range of concentrations to be evaluated, for the most part the high dose level, and the elements required in the selection of drug concentrations in the *in vitro* testing of drugs that are poorly soluble. An adequate rationale for the selection of the high dose is crucial as this is an area that is sure to be scrutinized by regulatory authorities when reviewing the test data.

ICH S2A provides a discussion as to the interchangeability of different test systems to evaluate *in vivo* genotoxicity of a test material. The bone marrow micronucleus test in rats or mice, the analysis of chromosomal aberrations in mouse or rat bone marrow cells, and the peripheral blood micronucleus test in mice were all considered adequate to assess the *in vivo* clastogenicity of the drug. The selection of the sex (either one or both) should be dependent on the pharmacokinetics and toxicity of the drug. Male rodents are sufficient provided there are no real gender-specific effects.

Perhaps the most important guidance provided in ICH S2A pertains to the interpretation of the test results. Since no assay will provide 100% predictivity, it is especially important to be able to differentiate between a true response and a false result (positive or negative). Criteria to be considered in the interpretation of the response(s) are provided in this ICH guidance.

3.5.2. Genotoxicity: a Standard Battery of Genotoxicity Testing of Pharmaceuticals (ICH Topic S2B)

ICH S2B provides excellent guidance as to the standard battery of tests to support clinical trials and the marketing application of a drug (Table 8).

Table 11 Safety Pharmacology Studies—Battery of Testing

Target organ system	Potential observations/endpoints
<i>Primary Organ Systems (Core Battery)</i>	
Central nervous system	Spontaneous locomotor activity, behavioral changes, coordination, sensory/motor reflexes, general anesthetic effect (i.e., pentobarbital sleeping times), effect on chemically induced analgesia and convulsion Functional observation battery (FOB) or Irwin's test are commonly conducted
Cardiovascular system	Blood pressure, heart rate, electrocardiogram (ECG), cardiac output
Respiratory system	Respiratory rate, tidal volume, airway resistance, blood gases and blood pH
<i>Secondary Organ Systems</i>	
Gastrointestinal system	GI transit time, drug effect on isolated ileum
Renal system	Urine volume, urine chemistry, glomerular filtration rate
Autonomic nervous system	Stimulation of autonomic nerves and measurement of cardiovascular responses, baroreflex testing

This standard battery should be followed without substitution of alternative tests unless there is valid scientific rationale for the substitution. There are three important points that ICH S2B addresses relating to the core battery of testing. First, the ICH panel of experts involved in the review of the different assays found a high level of congruence between the in vitro chromosome aberration testing and the in vitro mammalian cell mutagenicity testing of different drugs. For that reason, the guidance provides a recommendation that it is not necessary to conduct both testing schemes if the compound is negative in the other assays. Hence, if the compound is negative in the bacterial mutagenicity testing, the in vitro chromosome aberration testing, and in vivo mammalian genotoxicity testing, then in vitro mammalian mutagenicity testing is not required. Second, in accordance with the standard core battery of testing, the experts also recognized that there may be instances where bacterial mutagenicity may not be appropriate or may not provide sufficient information. In this instance, the in vitro mammalian mutagenicity testing should be conducted as part of the core battery of genotoxicity testing. Third, where conflicting test results are obtained in the core battery testing, the ICH guidance provides some recommendations for additional genotoxicity tests that can be added to the standard battery.

Lastly, ICH S2B provides specific procedural elements that can be followed in the conduct of the tests; for example, the use of RF tests as

sufficient replications of complete tests and the timing/durations of the exposure to the test drug. As this chapter is not intended to be a description of all methodology, the reader is referred to the guidance for protocol elements beyond those presented.

3.6. Carcinogenicity Study Guidelines

The historical norm for carcinogenicity studies has been to conduct such studies in both rats and mice for 2 years in duration. As more of these studies have been conducted, both in the support of pharmaceuticals and in the evaluation of potential environmental carcinogens, the investigation has focused on the relevance of animals studies to human cancer risk assessment especially given the long duration and large numbers of animals involved. The ICH has commented on the provided guidance in the area of carcinogenicity testing in three separate documents (16–18). These documents are summarized below.

3.6.1. Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals (ICH Topic S1A)

Carcinogenicity studies are generally required only in cases where the drug is to be administered over a chronic period of time to human subjects. In Europe and Japan, carcinogenicity studies were typically conducted when intended drug therapy was 6 months or longer. In the United States, drug administration for 3 months or longer generally required carcinogenicity studies. This ICH guidance defines the conditions under which carcinogenicity studies should be submitted to support approval of a drug.

Generally, drugs that are administered for 3 months continue to be administered for 6 months and beyond. The guidance has been harmonized to state that carcinogenicity studies should be performed for those drugs that are administered for at least 6 months. In those instances where the exposure may not be continuous for 6 months, but does occur intermittently over the lifetime of an individual, carcinogenicity testing should also be conducted. Carcinogenicity studies should also be considered for drugs given for less than 6 months, when which there exists risk factors suggestive of potential cancer risk in humans.

There are instances where despite chronic administration, carcinogenicity studies are not required. Drugs that are known to be genotoxic are hence presumed to be trans-species carcinogens. In these cases, no additional benefit would be gained by conducting long-term carcinogenicity studies, and these compounds are not generally tested in traditional 2-year bioassays. In addition, when the life expectancy of the target population is short, long-term carcinogenicity studies may not be needed. Anti-cancer drugs fall into this category. In instances where the drug is not systemically bioavailable (i.e., topical drug administration), there may not be a need to

conduct the studies unless there is cause for photocarcinogenic potential. Lastly, endogenous peptides or proteins do not generally require carcinogenicity testing.

With regards to timing, carcinogenicity studies are usually not required prior to initiation of any clinical trials. Rather, the studies are usually completed in time to support the application for marketing approval. For certain disease indications, the speed of approval is such that the carcinogenicity studies might be conducted and/or submitted as a post marketing approval commitment.

3.6.2. Testing for Carcinogenicity of Pharmaceuticals (ICH Topic S1B)

Of particular concern to the ICH is the large numbers of rats and mice used for carcinogenicity studies. This particular guidance poses the question as to whether one carcinogenicity bioassay would provide sufficient data to evaluate the cancer risk in humans. In conjunction with a single long-term bioassay, could alternative shorter-term studies be conducted that may better evaluate the carcinogenicity of a drug, the mechanisms involved, and determine whether the drug poses a cancer risk in humans, all with the added benefit of using fewer numbers of animals and a shorter duration of testing? The answer to this question is yes. The data from a chronic rodent bioassay in one species combined with other appropriate studies will enhance the assessment of carcinogenic risk in man. ICH S1B accordingly provides guidance as to selection of the rodent species (the rat in the absence of data favoring one species over another) for use in the standard carcinogenicity bioassay, additional short-to-medium length studies that will support the determination of cancer risk, and mechanistic studies that allow interpretation of a tumorigenic response and relevance of the response to man.

ICH S1B does provide a listing of potential carcinogenicity models that could be used as short-to-medium studies investigating potential carcinogenicity. These include initiation–promotion models of specific organ systems, transgenic mouse models including p53(+/-), Tg.AC, and TgHras models, and the neonatal rodent tumorigenicity model. A number of these studies are currently being evaluated for validity using pharmaceutical compounds of known carcinogenic potential, or lack thereof.

At this point, it is not clear as to which alternative shorter-term study(s) is favored by regulatory authorities. The available Summary Basis of Approval (SBA) documents discussed as case studies below (Section 5) generally followed the traditional long-term bioassays in rats and mice. However, these documents were part of development plans that existed prior to Step 5 approval of this ICH guidance by FDA in 1998. Nonetheless, the trepidation on the part of the toxicologist to conduct a shorter-term study in place of the chronic bioassay in the second rodent species is understandable if the results of such short-term studies consistently led regulators

to ask for more insight from the Sponsor by conducting the traditional rodent bioassay in the second species. Such a regulatory response would certainly be detrimental to the career of the toxicologist at his/her company as the timelines to drug approval could be drastically altered. The ICH S1B guidance does include the statement that “a long-term carcinogenicity study in a second rodent species is still considered acceptable.” Hence, some toxicologists may continue to recommend rodent chronic bioassays in two species to avoid the potential for equivocal/irrelevant data in a short-to-medium term study and subsequent delays in the critical path timelines. The pharmaceutical industry standards and practices will only become clearer as more SBA documents are released and the trends in the pharmaceutical industry and the regulatory environment become more evident.

3.6.3. Dose Selection for Carcinogenicity Studies of Pharmaceuticals (ICH Topic S1C)

The appropriate dose levels for carcinogenicity assays are crucial. Too high, and the dose level will need to be lowered mid-study or, worse still, the mortality is so great that entire treatment groups are terminated early (Section 5.1, Case Study 1: Celebrex). Too low, and one runs the risk of the criticism that the doses did not adequately address the carcinogenic potential of the drug. ICH S1C addresses these concerns by providing specific guidance on the proper selection of the doses for the chronic carcinogenicity assays, including the subchronic toxicity studies used to assist in the selection of dose levels.

Dosage selection, particularly the high dose, for cancer bioassays has been based on the MTD determined from subchronic toxicity studies, usually of 3 months in duration. In the United States, high-dose selection has been traditionally based solely on the MTD. In Europe and Japan, there has been the recognition that the MTD may far exceed the expected human exposure and not be relevant to assessment of human risk. Consequently, in addition to the MTD, an acceptable alternative to the MTD has been a high multiple of the maximum recommended human dose (>100-fold on a mg/kg basis). In keeping with one of the overall objectives to reach harmonization of study requirements, ICH S1C represents the culmination of mutually acceptable, rational and scientifically based criteria for selection of the high dose for carcinogenicity studies.

General guidances for the dose-RF studies to select the high dose for the carcinogenicity studies are provided in ICH S1C. Importantly, the ICH EWG agreed that a consensus on the use of toxicity endpoints other than the MTD would be difficult, and accepted the continued use of the MTD, determined in both males and females in a subchronic toxicity study, as an endpoint for high dose selection. The definition of the MTD in each of the three regions is provided in the “Notes” section of the guidance (18).

The role of the use of pharmacokinetics in the selection of the bioassay high dose level is a primary feature of the ICH S1C guidance. Systemic exposure may be especially important as an appropriate endpoint for non-genotoxic carcinogens that might be expected to have a threshold effect. In a retrospective analysis of the data from carcinogenicity studies for which there were sufficient rodent and human pharmacokinetic data, a review of systemic exposures, expressed in terms of the area under the concentration–time curve (AUC), was conducted. These systemic ratios were analyzed with respect to exposure and/or dose ratios for known or probable human carcinogenic pharmaceuticals (IARC Class I and 2A pharmaceuticals with positive rat findings). ICH S1C concludes from these evaluations that a relative systemic exposure ratio of at least 25 (rodent:man) is an acceptable pharmacokinetic endpoint to use for high-dose selection. Further, in order to establish the actual dose on a mg basis, comparisons between man and rodents of systemic exposures as a function of dose found that systemic exposures were better estimated by mg/m^2 rather than mg/kg . Accordingly, the guidance concludes that the high dose in the rodent carcinogenicity study should be at least 25-fold higher than the anticipated human clinical dose on a mg/m^2 basis.

Since systemic exposure is of critical importance in determining the potential high dose level, ICH S1C provides some specific guidances as to the conduct of specific studies (which may be the 3-month toxicity study with suitable evaluation of TK) that will be used to determine the systemic exposure ratio (note that this also assumes there is adequate human exposure data at anticipated dose regimen), including the use of doses across the anticipated carcinogenicity dose range and for durations of time sufficient to allow for any time- and repeated dose-dependent changes in pharmacokinetic parameters.

Once the selection of the high dose level for the carcinogenicity study is complete, the selection of the mid and low dose levels is somewhat more straightforward. Selection of the mid and low doses should take into account saturation of metabolism leading to a plateau of blood concentrations as well as potential saturation of absorption and elimination. Dose selection should also take into account alterations in rodent physiological parameters (e.g., the drug is anticipated to exert hormonal effects), as well as mechanistic information and the potential for threshold effects and human exposure and therapeutic dose.

An addendum to ICH S1C regarding the addition of a limit dose was added to the original guidance as ICH S1C(R). This addendum states that the limit dose for carcinogenicity testing should be $1500 \text{ mg}/\text{kg}/\text{day}$. This limit dose may be exceeded if the systemic exposure in animals resulting from such a dose does not exceed the human systemic exposure by at least one order of magnitude.

3.6.4. Additional Guidance Information and Protocol Elements in Carcinogenicity Studies

All three regions (USA, Europe, Japan) provide guidance with respect to protocol elements to be included in carcinogenicity studies (6,8,19). These recommendations are provided in [Table 9](#).

Each of the regions requires a group size of at least 50/sex/group. If the study involves daily oral intubation and the staff is inexperienced in conducting a study of this length, it may be useful to increase the group size (e.g., to 65/sex/group) to compensate for technical error. Some laboratories use 65/sex/group as a standard to ensure sufficient group size at the study termination, especially in mice, for long-term studies (2 years in duration).

A carcinogenicity study is not a chronic toxicity study, but rather a study only to assess the potential carcinogenicity of the compound. Thus, some elements that are part of repeat-dose toxicity studies are absent here (e.g., ophthalmology and ECG assessment). There is also some debate over the utility of clinical pathology determinations in the carcinogenicity bioassay. One preference is to include determinations as outlined in the FDA guidance in randomly allocated subgroups of 10 rats/sex/group. For mice, it is necessary to further separate animals into those being bled for clinical chemistry determinations and those being bled for hematology evaluation as the blood sample volume at a non-terminal time point is usually not of a sufficient volume to evaluate both clinical chemistry and hematology; this may be why the Japanese guidance asks only for a hematology evaluation. Given the inherent sensitivity of mice to carbon dioxide anesthesia and potential for death, combined with the desire to have sufficient group size at study termination, some investigators prefer not to collect blood samples for clinical pathology in a mouse carcinogenicity bioassay.

Finally, systemic blood exposures and pharmacokinetics are essential in the selection (and justification to regulatory authorities) of the dosage levels in a carcinogenicity bioassay. Hence, these data should be well defined prior to the conduct of the carcinogenicity study and there is no added value to include TK in these bioassays.

3.7. Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (ICH Topic S6)

ICH Topic S6 expressly addresses the preclinical safety evaluation of biotechnology drugs (20), but the recommendations are consistent with those provided for single-dose toxicity (ICH S4A), repeat-dose toxicity (ICH S4B), and timing and duration in support of clinical trials (M3). Development of the biologic product should follow these guidances. However, there are some potential differences in study design that are worthy of further discussion.

One likely difference lies in the selection of the animal species in which to conduct these single- and repeat-dose toxicity studies. Due to the nature of the species specificity of many biotechnology products, the selection of the relevant species may sometimes rule out commonly used laboratory animals (e.g., rodents and dogs), in favor of a species in which the biotechnology product has pharmacological activity, often primates. While the use of two animal species as discussed in the repeated-dose toxicity studies (see [p. 11](#)) is similarly recommended for biotechnology products, the identification of a single relevant species and toxicity studies in this single species may be justified. In fact, ICH S6 goes so far as to discourage the use of a non-relevant species in toxicity testing of the biotechnology product. In instances where no relevant species exists in which to test the human product, a homologous animal biotechnology product should be considered. The testing of the human product in a transgenic animal model in which the animal expresses the human target protein of interest is also an alternative, although a potential lack of the full spectrum of interacting human proteins in transgenic animals might make this model less than ideal.

Another potential difference is in the subchronic to chronic duration of the repeat-dose toxicity studies of biotechnology drugs. Many of the biotechnology products, by nature of their intended use, will be immunogenic when administered to animals on a repeated basis. Hence, the detection and characterization of an antibody response to the biologic are crucial. An antibody response may change the pharmacokinetics of the drug and may alter the pharmacological and/or toxicological profile of the biotechnology drug. If the antibody response is truly an immune response that neutralizes any pharmacological and/or toxicological activity of the biologic, then this may serve as a criterion for the early termination of a repeat-dose toxicity study and a justification for not conducting repeat-dose toxicity studies of longer length.*

While the immunogenic response of the animal to the biotechnology-derived product is important to characterize, ICH S6 does state that the routine tiered testing approach for immunotoxicology evaluation is not recommended for biotechnology products; the immunotoxicology tier approach has not been addressed by ICH but guidance can be found in the FDA Guidance for Industry entitled “Immunotoxicology Evaluation

*However, recent discussions by the author with FDA regarding potential immunogenicity of a therapeutic monoclonal antibody in a chronic study has led to the sense that this rationale for not conducting repeat-dose toxicity studies of longer length is being reconsidered. The FDA pointed out to the author that there are seeing increased evidence that animals can be tolerized to a therapeutic biologic by increasing the dose, increasing the dose frequency, or both. Consequently, if the repeat-dose toxicity study shows some evidence of dose tolerization, the FDA is likely to require additional longer-length studies.

of Investigational New Drugs” (10). For information on that tier testing approach, the reader is referred to that document.

In contrast to classic small molecule drugs, genotoxicity testing is not routinely required for biotechnology drugs. Nonetheless, as illustrated in the case studies presented later in this chapter, it is not uncommon to see the ICH recommended battery of genotoxicity studies conducted with biotechnology products. Carcinogenicity studies are also not generally performed on these biotechnology products, although the guidance does indicate circumstances where animal bioassays may be relevant. Similarly, reproductive toxicity testing is dependent on the biotechnology product and the intended clinical population. Again, the submission of such studies, or lack thereof, is illustrated in the case studies presented later in this chapter.

4. ADDITIONAL SAFETY TOPICS AND GUIDELINES

The preclinical toxicity testing of a drug was outlined in the previous section. Toxicity is one of the three major areas evaluated during preclinical development; the other two being pharmacology and pharmacokinetics (administration, adsorption, metabolism and elimination, hence the acronym ADME). For the purposes of completeness of the presentation of safety topics addressed by ICH, this section will briefly discuss the ICH guidelines to date in the realms of pharmacology and pharmacokinetics. The preclinical safety topics in these areas are provided in [Table 10](#).

4.1. Note for Guidance on TK: the Assessment of Systemic Exposure in Toxicity Studies (ICH Topic S3A)

The demonstration of systemic exposures to the drug product in toxicity studies is extremely important, perhaps best illustrated by the fact that this topic was addressed early on in the ICH proceedings (21). The incorporation of blood sample collection in animals, either the actual study animals or separate satellite groups, provides an evaluation and correlation of systemic exposure with toxicity endpoints and can validate the relevant selection of the animal species in the toxicity study. The TK data collected in animals can provide vital comparisons with the clinical data, allowing for assessment of potential risk and possible margin of safety of the drug product in humans. This ICH guidance provides strategies for incorporation of pharmacokinetic data collection in toxicity studies, termed TK. Excellent real-life examples of how these data have been collected in the conduct of toxicity studies and how the data are used, especially to design subsequent toxicity studies, are provided in the section on Non-clinical Drug Development Programs (see [Section 5](#)).

ICH S3A provides some guidance for the collection of TK data as a part of the toxicity testing discussed above; that is, single- and repeat-dose

toxicity studies, reproductive toxicity testing, genotoxicity testing, and carcinogenicity assessment. In single-dose toxicity studies, drug assays often are not developed. Consequently, TK is not a routine requirement in these studies. However, the ICH guidance does suggest that plasma samples be collected and stored for possible future analysis.

In the realm of repeat-dose toxicity testing, ICH S3A calls for profiling (collection of samples at more than four time points to allow for determination of the AUC) or monitoring (defined as collection of samples at 1–3 time points to estimate peak systemic concentration and time to peak) to be incorporated appropriately into the repeat-dose toxicity studies. Minimally, full-profile TK data should be collected at the start and towards the end of the treatment period of the first repeated-dose toxicity study; the study must be at least 14 days in duration. In practice, the standard toxicity package generally includes a 28-day toxicity study as a pivotal study and a full TK profile is routinely obtained in this study after the first and last dose of drug. The ICH guidance states that further collection of TK data in toxicity studies of different duration is not necessarily required if the dose levels and drug formulation are unchanged and that collection of TK data past 6 months of exposure is not essential. In practical terms, dose levels often change as the length of the repeat-dose toxicity study is increased. During repeat-dose toxicity studies of 3, 6, and/or 9 months, one should obtain full TK profiles after Days 1 and 28 at any dose levels for which the data do not exist. If the TK profile data have been collected in a previous study, it is appropriate to monitor (1–3 samples) on Days 1 and 28 as opposed to collecting a full AUC profile. In these 3, 6, and 9-month studies, monitoring is also recommended prior to dosing and at the estimated $C_{\max}$ at each 3-month time point. Examples of other approaches can be found in the case studies (Section 5).

For in vivo genetic toxicity testing, it may be useful to include monitoring of systemic blood concentrations to establish that the animals were exposed to the drug.

Carcinogenicity bioassays, as discussed previously in this chapter, are based on 13-week dose-setting studies to determine MTD, and these dose-setting studies should include determination of systemic exposures for comparison to human exposures. In the definitive carcinogenicity bioassay, the ICH guidance suggests monitoring at several occasions before 6 months. One suggestion is to monitor at pre-dose and at estimated $C_{\max}$ on Day 1, and at the end of 3 and 6 months.

The collection of TK data in reproduction studies will be based on the extent of data collected to that point. For example, if exposures have been documented in repeat-dose at toxicity studies at similar dose levels, it is not essential to include TK data collection in the fertility and the peri-/postnatal studies if conducted in rats. However, for the embryo–fetal development studies in rats and rabbits, it may be prudent to include TK data collection

since there is the possibility that the pharmacokinetic profile may be different in pregnant animals. These TK data are usually collected in satellite pregnant animals, to avoid any influence of the blood collection procedure on the data in the main study animals. Since systemic exposure data collected in the RF studies are used in the selection of doses, it is not a requirement that these data again are generated in the definitive study if the conditions and dosing regimen in the study are not different from those of the RF study.

4.2. Pharmacokinetics: Guidance for Repeated-Dose Tissue Distribution Studies (ICH Topic S3B)

This ICH guidance is a short list of circumstances under which repeat-dose tissue distribution studies should be considered in the preclinical development of a drug (22). Repeat-dose tissue distribution studies should be considered in cases where: (1) the tissue half-life is much greater than the plasma half-life; (2) steady-state levels with repeated-dose studies are significantly higher than predicted from single-dose studies; (3) histopathological changes are observed for which tissue distribution studies may clarify the interpretation of the findings; and (4) the drug is targeted to a specific tissue in the body. The dosing duration specified in the guidance is from 1 to 3 weeks. The guidance reiterates that for most drug development programs single-dose tissue exposure tissue distribution studies are sufficient for regulatory authorities.

4.3. Safety Pharmacology Studies for Human Pharmaceuticals (ICH Topic S7A)

This guidance by the ICH is intended to provide a specific harmonized guidance for the scope of safety pharmacology studies to be conducted for marketing approval (23). Safety pharmacology studies can be thought of as studies that evaluate the unintentional potential pharmacological effects of the drug on organ systems; that is, the pharmacological effects beyond those which the drug was designed to possess. In addition to this ICH guidance, the Japanese authorities have a well-written chapter that provides guidance for safety pharmacology testing (the chapter is entitled "Guidelines for General Pharmacology Studies" with the definition of General Pharmacology in the JMW guidelines being roughly equivalent to the definition of Safety Pharmacology in the ICH guidance) (8). For a comprehensive understanding of the study designs and protocol elements, the reader is encouraged to consult these Japanese Guidelines along with the ICH guidance document.

The package of safety pharmacology studies will encompass evaluation of the effects of the drug product on the major organ systems in the

body; the cardiovascular system, the central and autonomic nervous systems, the respiratory system, the gastrointestinal system, and the renal system. The ICH identifies a core battery of testing to encompass the effects of the drug on the central nervous system (CNS), the cardiovascular, and the respiratory systems. ICH Topic S6 also recommends that this core battery of testing be utilized in the development of biotechnology-derived products. Depending on the class of compound and pharmacological mode of action, safety pharmacology testing can also include, or exclude with sufficient rationale, effects on the gastrointestinal, peripheral nervous system, and renal system. Examples of observations and endpoints that can be used to evaluate potential drug effects on each organ system are provided in [Table 11](#). The safety pharmacology testing in the core battery should be conducted in GLP compliance. Supplemental testing should be conducted in the spirit of GLP regulations to the extent feasible since absolute compliance to the GLP may be difficult.

As can be seen from the listing in ? there are a number of safety pharmacology endpoints that can be incorporated as components of the toxicity testing studies with potential cost savings both in terms of money as well as in total numbers of animals used. Motor activity, behavioral changes, coordination, and body temperature are all endpoints that are easily captured in the conduct of single- and repeat-dose toxicity testing. Similarly, urine volume and urine chemistry should also be components of repeat-dose toxicity testing.

The following case example illustrates the potential value of including safety pharmacology endpoints in toxicity testing. A study director was assigned a drug that was about to enter Phase III trials. At the end of the Phase II meeting with FDA regulatory authorities, concerns were raised over the potential cardiovascular effects of the drug, an intravenous anti-infective molecule, even though there was no apparent reason why any such effects might be observed. This FDA concern would have easily been addressed had the repeat-dose toxicity study in dogs included collection of ECG data in all dogs at all dose levels at various time points during the study. However, the previous study director believed that such testing would not add any value (and was being instructed from management to keep cost down) since this particular class of drug was not known to have any cardiovascular effects. Therefore, full ECG testing was not performed. Instead, ECG testing was done in just two control and two high dose dogs at one time point. Subsequently, the FDA requested a full cardiovascular safety pharmacology study with collection of ECG parameters over a 24-hr period before and after dose administration, in the end costing money, time, and the use of extra animals.

ICH S7A provides some guidance as to the timing and applicability of safety pharmacology testing in the drug development process. Safety pharmacology testing is not required for locally applied agents and/or instances

where systemic exposures are anticipated to be minimal. Studies with cytotoxic drugs in cancer are usually excluded from any safety pharmacology testing. Biotechnology products with highly specific targets and mechanisms of action may also be exempt from safety pharmacology testing, although biotechnology products with less-specific targeting or unknown mechanisms of action should have the core battery assessment of testing. With regards to timing, safety pharmacology testing in the core battery of systems should be completed prior to administration of the drug in the clinical setting.

4.4. Safety Pharmacology Studies for Assessing the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals (ICH Topic S7B)

The high profile for the cardiovascular system in the assessment of safety pharmacology is emphasized with this ICH guidance extending the guidance discussed in ICH Topic S7B to specifically identify and assess the risk of potential cardiovascular effects, specifically effects on the QT interval (24).

ICH S7B provides general recommendations for the testing strategy to evaluate risk of a drug product to cause a prolongation of the QT interval in man. The guidance calls for evaluation in four particular areas. Number 1 is the evaluation of the pharmacological class to which the drug product belongs and whether this class is known to possess cardiovascular effects. Number 2 is an evaluation of the drug effects in an ionic current assay in vitro (e.g., isolated animal or human myocytes, cultured cardiac cell lines). Number 3 is an evaluation of action potential parameters in isolated cardiac preparations or alternatively, the measurement of specific electrophysiological parameters indicative of action potential duration in animals. Number 4 is an in vivo QT assessment. This assessment should be a component of the core battery cardiovascular study conducted as part of the safety pharmacology evaluation described in ICH S7A. An investigator can expand the scope of the in vivo QT assessment to include regional information relating to ventricular repolarization, and thereby satisfy testing in area Number 3. Each of these evaluations should be complete prior to initiation of clinical trials.

ICH topic S7B also provides extensive guidance and protocol elements of investigational test systems to address drug effects in each of the areas, numbers 2, 3, and 4, described above. A discussion of each of the test systems in the ICH guidance is beyond the scope of this chapter and the reader is referred to the ICH S7B guidance document (24).

5. NON-CLINICAL DEVELOPMENT PROGRAMS

This section presents some case studies of drug development programs that have been reviewed by the regulatory authorities in the United States and/or in Europe and approved to support the marketing approval of the pro-

duct. Under the Freedom of Information Act (FOIA), and subsequent amendments, federal agencies including the FDA, place FOIA materials (including drugs and biologics approvals) in publicly accessible electronic reading rooms. The internet address for FDA Summary Basis of Approval (SBA) documents in CDER is www.fda.gov/cder/approval/index.htm and the address in CBER is www.fda.gov/cber/products.htm. All of the information discussed in each of the following drug development case studies is part of the Public Record and can be found in the approval documents at one of these sites. The SBA documents are invaluable in providing insight into current regulatory practices and development programs and the reader is encouraged to review the regulatory review documents for other drugs and biologics. In addition, analogous review and approval documents are available for drug products approved for use in Europe; these can be found on the EMEA website at www.emea.eu.int/index/indexh1.htm.

In reviewing different non-clinical drug development programs, it becomes apparent that the regulatory guidances are not to be used as a cookbook from which to design in ubiquity one drug development program after another. Each drug development program is likely to be unique and full of particular challenges such that, on some days, one may yearn for just one “simple” drug in development. The old adage “don’t miss the forest for the trees” is quite apt for non-clinical drug development if one considers the forest to be the non-clinical plan of studies to support the drug and the trees to be each study. It is important to keep in mind that the purpose of the non-clinical studies is not to provide a checklist of studies that have been conducted with the resulting toxicities in the animals. Rather, the purpose of the non-clinical studies is to support the safe dosing of humans with the new drug in clinical trials.

It is when one realizes that non-clinical toxicity studies are conducted with the goal of being supportive to the assessment of the safety in man that one can fully utilize the guidelines. The most appropriate package of non-clinical toxicity studies will be the one that provides the best extrapolations of toxicities in animals to potential toxicities in humans. This mindset of thinking makes clear that the best non-clinical toxicity program is one that uses the most appropriate animal species, doses, dosing regimen, duration of dosing, and study endpoints to predict effects in humans.

When assessing treatment-related effects, the following factors should be used to evaluate the significance of differences between treated and control groups:

- dose-related trends;
- reproducibility;
- related findings;
- the magnitude and types of differences
- occurrence in both sexes.

Finally, before proceeding to the presentation of case studies, one last item bears mentioning. As most persons in the pharmaceutical industry will point out, when you buy a medicine you are not so much paying for the cost of the ingredients used to prepare the drug as you are paying for the package insert that comes with the medicine. Consequently, all drug development should be geared towards the contents on the label. It is important that the package of toxicity studies address any labeling concerns for marketing the drug to physicians and the general public.

As an example, a package insert contains a use-in-pregnancy rating system to classify the risk to the fetus. These risks of fetal harm are divided into Category A, B, C, D, and X. Category A is defined as "Controlled studies show no risk" in which adequate, well-controlled studies in pregnant women do not demonstrate a risk to the fetus. Category B is defined as "No evidence of risk in humans" in which animal studies have been conducted and show no fetal risk but there are no controlled studies in pregnant women. Category C is defined as "Risk cannot be ruled out"; in this category, animal studies and human-controlled studies are lacking or animal studies have shown a risk to the fetus and there are no human-controlled studies. In this category, drugs should only be given if the potential benefit outweighs the potential risk to the fetus. Category D is defined as "Positive evidence of risk" where there is evidence of human fetal risk, but the benefits for use in pregnant mothers may be acceptable despite the risk. Category X is defined as "Contraindicated in pregnancy" where studies have demonstrated fetal abnormalities and the risk of drug use outweighs any potential benefit. In some instances, reproductive toxicity testing may not be required for marketing approval. However, a competitor product may have a Category C label because no reproductive toxicity studies were conducted. It may be desirable, then, to conduct the reproductive toxicity testing in order to use a Category B label (assuming no adverse effects were detected). Similarly, additional non-clinical studies that may be important for inclusion in the product label insert should be considered in the context of the development program.

5.1. Case Study 1: CelebrexTM (NDA 20-998, Celecoxib, SC-58635)

Celebrex is a well-known drug approved for the treatment of acute and chronic signs and symptoms of rheumatoid arthritis and osteoarthritis and management of acute and chronic pain. The drug is a new chemical entity (NCE), a non-steroidal anti-inflammatory inhibitor of the cyclooxygenase 2 (COX-2) enzyme, and as such was reviewed by CDER. Celebrex is provided in capsules at strengths of 100 and 200 mg and is to be administered orally. The Sponsor, G.D. Searle & Co., submitted the NDA for Celebrex for review on June 29, 1998 and the Pharmacology review was

completed within 6 months on November 24, 1998. The SBA can be found under the Freedom of Information act at: www.fda.gov/cder/approval/index.htm. Given the widespread usage of the drug, it is interesting to examine the studies used to support the marketing application in the United States. Indeed, for the novice toxicologist, the list of studies evaluated in the SBA provides an invaluable blueprint of those studies that would likely be required for support of a standard NCE.

The list of toxicity studies in support of the NDA for Celebrex is given in [Table 12](#). The studies listed were standard to support chronic administration of a drug to a potentially large population of patients. Rats were the primary rodent species examined, while dogs were the primary non-rodent species. It is interesting to note the different forms of the drug administered in these toxicity studies. In rats, the drug was administered by oral gavage of a methycellulose/Tween 80 suspension, whereas in dogs, capsules were administered. In mice, the drug was administered in the diet.

For mice, the studies described are classic studies that one would conduct in series with the overall end goal being the evaluation of carcinogenicity of the drug. The first study investigated whether administration of the drug in the diet could provide adequate systemic exposures and, perhaps, a consistent estimation of a target daily dose. The advantage of diet administration is obvious in not having to oral gavage dose several hundred mice daily for 2 years, a task requiring highly skilled animal technicians to avoid accidental dosing deaths. The target doses chosen in the 2-week study were 100, 300, 1000, and 3000 mg/kg/day, with a group size of 10/sex. Males were more sensitive than females to the toxic effects of the drug, correlating with greater systemic exposure in this gender; the NOAEL was 100 and 300 mg/kg/day for males and females, respectively. The dose levels in the 13-week study (to define the doses for the long-term carcinogenicity study) were consequently set at 0, 75, 150, and 300 in males and 0, 150, 300, and 1000 mg/kg in females with a group size of 20/sex. Both 2-week and 13-week studies included a large number of satellite mice for TK purposes; the TK samples were collected on Days 1, 45, and 87 in the 13-week study. The outcome of the 13-week study was a NOAEL of 150 mg/kg/day in females and < 75 mg/kg/day (the low dose) in males; systemic exposures were 2-fold higher in males at equivalent target dietary doses. The major target organ in the study was the gastrointestinal tract. One comment here on dose-setting. It would have been beneficial to identify a NOAEL in males to have a greater degree of confidence in the doses for the carcinogenicity bioassay.

The carcinogenicity study in mice used target doses of 0, 25, 50, and 75 mg/kg/day (diet) in males and 0, 50, 100, and 150 mg/kg/day in females. The numbers of animals were 90/sex/group, far in excess of the 50/sex/group suggested in the ICH guidance, but the increased numbers allowed for a scheduled interim necropsy at 1 year (such an interim necropsy is

Table 12 Toxicity Studies^a Submitted to Support Marketing Approval of CelebrexTM in the USA

Mice	Rats	Dogs	Other studies
2-week diet admix toxicity study; included TK	Acute oral toxicity studies; some included TK	Single-dose oral toxicity study	Acute limit study oral monkeys
13-week diet admix toxicity study; included TK	4-week oral toxicity study with 4-week recovery; included TK	4-week oral toxicity study with 4-week recovery; included TK	Teratology, embryo-fetal (2 RF and 1 definitive studies)—oral rabbits
104-week diet admix carcinogenicity study	13-week oral toxicity study with 4-week recovery; included TK	13-week oral toxicity study with 4-week recovery; included TK	Ames assay
	26-week oral toxicity study; included TK		In vitro mutagenicity in CHO
	104-week oral carcinogenicity study	52-week oral toxicity study with 4-week recovery; included TK	In vitro chromosome aberration in CHO
	Fertility, early embryonic development (three separate studies)—oral	7-day exploratory i.v. toxicity study; included TK	In vivo rat bone marrow micronucleus assay
	Embryo-fetal development (2 separate studies)—oral		Antigenicity
	Perinatal/postnatal development study—oral		Guinea pig maximization
			Primary irritation—dermal and ocular in rabbits

^aNot listed in this table are studies conducted with chemical intermediates in the production process; these were an acute oral toxicity study in rats, primary dermal and ocular irritation studies in rabbits, a guinea pig maximization test, and an Ames assay.

not required and is somewhat unusual). There were changes in dose levels throughout the study that were reflective of the close monitoring of mice during the study by the Study Director and, although not ideal, reflected the need for flexibility in changing the dose levels in a study as necessitated by the animals rather than risk the entire study by stubbornly adhering to an original design. All dose levels for both males and females were halved at Week 19 due to excessive toxicity. Oddly enough, the high dose (and only the high dose) for females was returned to the original 150 mg/kg/day at Week 23. The interim necropsy at Week 52 revealed no marked treatment-related findings at any dose level. Nonetheless, at Week 80, all high dose animals of both sexes were terminated due to poor survival with gastrointestinal toxicity being the main finding. To maintain study validity and provide control animals at this unscheduled necropsy and at scheduled termination, the Study Director ingeniously terminated the control satellite PK mice to provide control-matched tissues. The remaining mid- and low-dose group mice survived to 2 years, with gastrointestinal changes observed histopathologically in only a minority of animals and no evidence of carcinogenicity. Overall, these studies conducted in mice are a good illustration of the difficulties in dose setting with a compound showing a steep dose-response curve and of the real-time adjustments in study design necessary to gain the most complete picture of toxicity that is possible with such a compound.

The standard toxicity studies were conducted in rats for Celebrex, with some additional unusual design elements. The 4-week toxicity study consisted of 6 groups of 10–15/sex allocated to dose groups of 0, 20, 40, 80, 400, and 600 mg/kg/day; the extra 5/sex were animals assigned to a 4-week recovery period. Interestingly, the study included TK evaluations in satellite animals as would normally be recommended in a 4-week study, but only in the mid and high doses and only on Day 31 as opposed to the first and last day of the study. There was very little toxicity in this study. The NOAEL of 80 mg/kg in males and 400 mg/kg in females was not driven by marked toxicities across the animals in these groups, but by the death of one male at 400 mg/kg and one female at 600 mg/kg; these two rats had marked gastrointestinal lesions.

The 13-week rat study was a standard toxicity study with groups of 25/sex allocated to dose groups of 0, 20, 80, and 400 mg/kg/day; 10/sex/group of which were assigned to a 4-week recovery period. This 4-week recovery period is not a standard design element in a study of this length, but most likely reflects the fact that there were no toxicities observed in the 4-week study for which reversibility needed to be assessed. The study, in terms of TK evaluation, was unusual in that a radiolabeled version of the drug was administered on the TK profiling Days 1, 37, and 86. Hence, the TK component of the study included evaluation of urine and feces along with measurement of drug metabolites; such PK studies with radiolabeled

drug are usually conducted separately from the toxicity studies and for much shorter study durations. In contrast to the greater systemic exposure in male vs. female mice, in rats females were found to have the higher exposures relative to similarly dosed males. The NOAEL in this 13-week rat study for both sexes was the highest dose of 400 mg/kg/day. One could argue that the high dose level could have been increased in this study, but the highest dose administered in the 13-week study was based on death-related MTD of 400 and 600 mg/kg in the previous 4-week study.

The 26-week rat study utilized the same dose regimen and group size of 25/sex/group as the 13-week study. Again, this study included a 4-week recovery period with likely a similar rationale for the inclusion of the design element. Like the 13-week study, this 26-week study also included satellite groups that received radiolabeled compound on Days 1 and 177; full TK profiles were collected following dosing on these days along with evaluation of urine and feces. The lack of TK data collection at interim time points in this 26-week study demonstrates that one does not need to collect redundant data; that is, there is no need to collect TK data at the same time points as in a previous shorter duration study when the dose levels are the same. As with the previous studies, females were found to have higher systemic exposures to the drug. These higher exposures correlated with the gastrointestinal injury and death of females at the 80 and 400 mg/kg/day dose levels; the NOAEL in males was again the high dose of 400 mg/kg/day.

There is one additional observation in the repeat-dose oral toxicity studies in rats that bears mentioning for toxicologists designing study protocols. In these rat studies, several deaths were attributed to technician errors in the dosing procedure. These errors can only be confirmed when the protocol states that satellite (TK) animals will be necropsied in the event of an unscheduled death. These deaths are not uncommon and without necropsy results it may not be possible to rule out a drug-related toxicity. It is in the best interest of the study Sponsor to ensure that all unscheduled deaths will be investigated by a protocol mandate.

The oral rat carcinogenicity bioassay was a 2-year study with 80 rats/sex/group allocated to dose groups of 0, 20, 80, and 400 mg/kg. A cohort of each group (10/sex/group) was terminated at an interim necropsy at Week 53. The rationale for the increased group size (> 50/sex/group recommended by ICH) cannot be ascertained from the SBA document, but was perhaps reflective of the uncertainty in dose selection and the interim necropsy. It should be noted that the treatment initiation date for this carcinogenicity bioassay was the same month as the 26-week repeated-dose toxicity study; this timing itself is not unusual in that these studies are often run in parallel rather than in sequence for the sake of time. Consequently, the dose levels in the carcinogenicity bioassay in rats were determined by the data obtained in the 4-week and 13-week repeat-dose studies as in the mice; the toxicities in females at 26 weeks were not a factor in

dose selection in the carcinogenicity study. For reference, the systemic exposures in males in the high dose of 400 mg/kg/day, based on TK data from the 13-week study, were 5-fold and 10-fold greater than systemic exposures expected in humans receiving the expected clinical doses of 400 and 200 mg, respectively. The experimental design elements of an interim necropsy and the inclusion of satellite animals to provide full TK profiles at Weeks 1, 26, 52, and 78 are unusual and not normally seen in a carcinogenicity bioassay. The results indicated the lack of carcinogenic potential for the drug. Before proceeding, there is one last note regarding the test article. There is no absolute requirement that the study utilizes the same lot of drug across all studies. In the rat study, different lots of test compound were used during the course of the bioassay.

The oral toxicity studies in dogs were based on complicated, and not entirely conventional, study designs. In the 4-week toxicity study, groups of 4/sex were allocated to receive 0, 25, or 50 mg/kg daily for 4 weeks. Additional groups of 8/sex received 0, 100, or 250 mg/kg daily for 2 weeks followed by 2 weeks of recovery. There was an interim necropsy of 4/sex/group at Day 17 with the remaining animals terminated at Day 29. This is an atypical study design and may reflect a desire to really push the highest doses to produce toxicity and evaluate the reversibility of such toxicities. As would be standard protocol for a 4-week toxicity study in dogs, blood samples were collected for TK at numerous time points following dosing on Days 1 and 28 to provide full drug profiles. An additional unusual study design element was the inclusion of satellite TK groups of 2/sex administered 25 or 100 mg/kg/day for 28 days, with radiolabeled drug given on Days 1 and 28 to allow identification of metabolites and quantitation of elimination in urine and feces; something seen previously in the rat studies. Gastrointestinal findings were the major toxicity in this 4-week study, again with a steep dose-response curve; the NOAEL was 25 mg/kg/day while doses of 50 mg/kg/day exceeded the MTD with moribundity and death observed in dogs at these higher doses.

There were additional pathological findings of interest to the FDA reviewer in the 4-week dog study. Interdigital pyoderma and focal areas of subcutaneous inflammation (cellulitis) with necrosis and abscess formation in the caudal-ventral neck were seen in several treated dogs. The Sponsor pointed out that interdigital pyoderma is a common bacterial infection of the pedal skin of short-haired breeds of dogs and these observations were not noted to be dose-dependent. Therefore, the Sponsor concluded that these findings were not associated with administration of the test article. However, the FDA reviewer did not agree since these findings are not commonly observed in the laboratory setting. Moreover, the FDA reviewer also had knowledge that similar cutaneous lesions were observed in dogs administered other COX-2 inhibitors. Also observed in the study was a perivascular/periventricular lymphocytic inflammation in the brains of several dogs

that was seen with a slightly higher incidence in the drug treatment groups. The FDA reviewer noted that a relationship to treatment could not be ruled out without additional study to determine whether there was a relationship to drug treatment or whether the changes were due to an underlying viral inflammation or another causes. It is worth noting that these findings did not adversely affect the drug development or drug review process by requiring an additional mechanistic study since these findings were observed only at doses in excess of the MTD.

The 13-week dog study also used an elegant, non-standard study design to incorporate multiple endpoints in the single study. Given the steep dose-response curve observed in the 4-week study, this study design included a dose group receiving single daily doses of the NOAEL of 25 mg/kg and dose groups receiving twice daily administration at 0, 7.5, 12.5, and 17.5 mg/kg; $n=4/\text{sex}/\text{group}$. Note that the twice daily administration of 12.5 mg/kg/dose allowed direct comparison of 25 mg/kg/day given all at once or in two divided doses. The higher 17.5 mg/kg dose allowed one to ascertain whether the NOAEL could be increased by twice daily dosing, keeping in mind the mortality observed when one approached 50 mg/kg/day. The control and high dose groups also included an additional 2/sex/group for a 28-day recovery period. The inclusion of recovery groups in a study of 13 weeks is likely reflective of the change in dosing regimen from the previous 4-week study. Similar to the 4-week oral toxicity study, there were two satellite groups of 3/sex/group that were administered radiolabeled drug on Days 1, 39, and 88. However, in contrast to the 4-week study, TK evaluations were only conducted on these satellite dogs and not on main study animals. To maximize the information in this study, the Sponsor also included in vitro metabolic activity in the livers of control and treated main study dogs. These additional PK add-ons were quite valuable with the Sponsor demonstrating distinct populations of fast and slow metabolizers of the drug. No remarkable toxicities were observed in this study indicating a NOAEL in excess of 17.5 mg/kg twice daily and 25 mg/kg once daily.

The 52-week dog oral toxicity study had the same treatment groups as in the 13-week study. The group size was 8/sex/group with an additional 4/sex/group assigned to a 4-week recovery for the control and high dose groups. Again, the inclusion of recovery groups is unusual but likely to allow for recovery of effects that might have been seen with this more chronic drug administration. Of the 8/sex/group, half were terminated at an interim necropsy at 26 weeks. An interim necropsy on a chronic dog study is atypical but likely reflects a desire to initiate a clinical trial of up to 26 weeks and the need for this supporting non-clinical data prior to completion of 52 weeks of chronic animal dosing. An additional 4/sex/group were allocated to satellite TK groups to receive 7.5 and 12.5 mg/kg twice daily, with radiolabeled drug administered on Days 1, 176, and 358. As in

the 13-week repeat-dose toxicity study, there were no remarkable drug-related toxicities at the highest dose levels of 25 mg/kg once daily or 17.5 mg/kg twice daily. This lack of toxicity at any dose level would be a potential criticism in both studies, in that the major objective of the experimental design is to allow elucidation of dose levels at which toxicity occurs along with a dose-response relationship and identification of a NOAEL dose. However, in this instance the MTD was established as 50 mg/kg/day in the 4-week oral dog toxicity study, and consequently to have administered that dose level in studies of longer duration would not have been prudent as such a dose level would have been overly toxic. Hence, the Sponsor was prudent in the selection of dose levels for the 13- and 52-week studies.

In summary, the oral toxicity studies in dogs illustrate several important points. First, it is possible to expand normal toxicity studies to encompass a variety of endpoints that one might normally associate with separate studies by independent groups of researchers. Second, the results of chronic toxicity studies are valid even in the absence of observed toxicities, provided that the MTD has been established in a repeated-dose study of shorter duration. Third, one can report findings (in this case histopathological findings in the 4-week dog study) that do not require a full explanation of definitive causality to drug treatment and/or mechanistic interpretation and extensive (often fruitless) investigation down blind trails.

The Sponsor is obligated under certain circumstances, to more fully characterize toxicity in animals and the potential toxicity risk in humans. Such was likely the case in the 7-day repeat-dose intravenous (i.v.) study in dogs conducted in 3 dogs/group (across sex randomly) at doses of 0, 15, and 40 mg/kg/day. This study was carried out to determine the relationship between the gastrointestinal activity of the drug and the systemic exposure. If the gastrointestinal activity were merely a local effect, one would likely not see such toxicity with i.v. administration. This mechanistic study evaluated only gastrointestinal histopathological and biochemical changes with the end result being a greater understanding of the gastrointestinal effects of the drug. To this end, the Sponsor demonstrated gastrointestinal effects consistent with the known properties of the drug.

The dog was the primary non-rodent species used to characterize the toxicity of the drug. Interestingly, a single-dose study was conducted in three monkeys/group administered 25 or 250 mg/kg. This is a peculiar study because the objective was only to determine the limit of lethality over a 14-day period following a single dose; there was no clinical pathology evaluation, no necropsy, and the PK sampling was limited to 2 time points on Day 1. Hence, this study would have been of limited value in the overall marketing application and the purpose is not known. The timing of the study indicated that it preceded the definitive single-dose studies in rats and dogs and it may have been simply an exploratory screening study.

The study is reported here only to remind the reader that all studies performed with a compound are required to be submitted in the marketing application, even if they are not conducted with expressed intent to support clinical trials.

The standard ICH package of studies was conducted for Celebrex although there were some redundancies with repeated studies. The design elements of the studies are briefly covered here to illustrate concordance with the ICH guidance and rationale for repeated studies. Of particular note is that of the studies listed in the ICH guidance, the embryo–fetal development (ICH 4.1.3) and the male and female fertility (ICH 4.1.1) studies were initiated at roughly the same time period, while the peri-/postnatal development (ICH 4.1.2) was initiated roughly a year later. This timing is fairly typical in that the embryo–fetal development studies are frequently completed early in the non-clinical development often prior to initiation of human clinical trials, whereas the peri-/postnatal development study is often completed later in the non-clinical development of a drug, being required primarily for labeling and not for safety in clinical trials.

There were three Fertility/Early Embryonic studies (ICH Study 4.1.1) conducted with Celebrex. All three studies used a group size of 25 rats/group. The first two were conducted after the 4-week repeat-dose rat study was completed and hence, likely relied on that study for dose levels. In the first study, dose levels were 0, 60, 300, and 600 mg/kg/day and males were exposed for 4 weeks prior to mating; note that the study was conducted prior to the ICH S5B(M) guidance. Females were exposed at these same dose levels from 2 weeks prior to mating to gestation Day 7. There were no effects on male fertility, but there were decreases in live fetuses and implantation sites and increased post-implantation losses in females at all dose levels. Subsequently, a second study was conducted only in females at dose levels of 0, 15, 30, 50, and 300 mg/kg/day, again for 2 weeks prior to mating to gestation Day 7. In this second study, the Study Director included an extra fourth dose group, probably in an attempt to bracket a range of exposures that provided the highest possible NOAEL; that is, an investigator could choose a very low dose (say 1 mg/kg/day) to definitely obtain a NOAEL but such a lower NOAEL would provide less of a margin of safety in humans. Similar results were observed in this study at higher doses, but the NOAEL was established at 30 mg/kg/day. Finally a third study was conducted in females to assess reversibility of the effects. In this study, females were administered 0, 60, or 300 mg/kg for 14 days followed by a 14-day recovery period prior to mating. No adverse treatment-related effects were observed in this study. Hence, the three rat studies established NOAELs of 600 mg/kg/day in males and 30 mg/kg/day in females and demonstrated that effects in females were reversible even up to a high dose of 300 mg/kg/day.

The peri- and post-natal development study was conducted at dose levels of 0, 10, 30, or 100 mg/kg administered from gestation day 6 to days 21–23 post-partum to groups of 25 rats/group. It is worth mentioning some design elements although there were no effects on the F1 and F2 generations. At Day 4 post-partum, the litters were culled to 8 pups, 4 males and 4 females (recall that litter culling is still under scrutiny by ICH in 2003). Physical development in the F1 pups was assessed as pinna unfolding, tooth eruption, and eye opening and reflexological development was assessed with geotaxis testing and the startle response testing. For the adult F1 generation, one male and one female were selected from each litter on Day 21 post-partum. Consequently, the behavioral testing and reproductive testing of the F1 generation were conducted in the same animals as suggested in the ICH guidance. Physical development of the selected animals was evaluated as day of vaginal opening and day of preputial separation and visual function as papillary closure and visual placing on Day 21 post-partum. Behavior performance was evaluated by motor activity in “figure 8” mazes on Days 35 and 60 post-partum, auditory startle habituation on Day 55 post-partum, and performance in “E” water maze on Days 60 and 70 post-partum. Mating of the F1 generation was initiated on Day 85, the females were allowed to deliver the F2 generation, and the F2 generation was terminated on Day 5 post-partum.

Two embryo–fetal development studies (ICH Study 4.1.3) were conducted in rats. Both studies used doses of 0, 10, 30, and 100 mg/kg/day on gestation days 6–17. The first study used 20 rats/group with additional satellite pregnant rats for TK profiles on gestation days 6 and 16. This study found a slight decrease in live fetuses at 100 mg/kg/day and increased incidences of wavy ribs at 30 and 100 mg/kg/day. Hence, the NOAEL for fetal development was 10 mg/kg/day. The second study was conducted approximately two years later and used a larger group size of 30/group with additional satellite pregnant rats for TK profiles on gestation days 6 and 17. This group size of 30/group was significantly larger than the group size of 16–20 recommended in the ICH guidance, but may reflect a desire to confirm with a larger group size whether or not the skeletal effects observed in the first study were definitively related to drug treatment. Unlike the first study, there was no increased incidence of wavy ribs or a decrease in number of live fetuses, but there was a dose-related increased incidence of diaphragmatic hernia at 30 and 100 mg/kg/day. Consequently, the NOAEL for fetal development was again 10 mg/kg/day. With these results, the drug is labeled Category C in the package label insert. The increased incidence of skeletal abnormalities is detailed and the increased incidence of diaphragmatic hernia noted in the package insert.

The embryo–fetal development studies in rabbits (ICH 4.1.3) consisted of a dose RF study, a pilot study, and the definitive study. The RF study consisted of 6 animals per group allocated to receive 0, 6, 30, 60, 300, or

600 mg/kg/day on gestation days 7–18. In this RF study, blood samples for TK analysis were collected from the study animals on gestation days 7 and 18. A necropsy was conducted on gestation day 29 and fetuses were examined externally. The study found significant maternal and embryo–fetal toxicity at 300 and 600 mg/kg/day. The pilot study was conducted to further define the high dose for the definitive study. Rabbits ($n = 2/\text{group}$) were dosed on gestation days 19–23 or 21–25 at doses of 200, 400, or 600 mg/kg/day. Based on this study, the drug was considered toxic at 600 mg/kg/day. In the definitive study, 20 rabbits/group (litter size consistent with ICH Study 4.1.2 recommendations) were allocated to receive 0, 60, 150, or 300 mg/kg/day from gestation days 7 to 18. Again, blood samples for PK evaluation were collected on gestation days 7 and 19. The collection of blood samples from main study animals is not necessarily the norm as some investigators fear that collection of the blood samples from main study animals might affect the study outcome. Therefore, it is not surprising to see separate satellite TK groups of pregnant animals that are terminated after the final day of dosing with no evaluation of fetal parameters, with the possible exception of drug concentration determinations in fetal tissues. A slight dose-dependent increase in skeletal abnormalities was observed at 150 and 300 mg/kg/day.

The ICH recommended battery of genetic toxicity tests were conducted with Celebrex and all assays were conducted as outlined in the ICH S2A and S2B guidances. The Ames bacterial mutagenicity assay included six concentrations over 2 logs of concentrations. The highest concentrations were precipitating, and colony counts were not determined. The highest non-precipitating concentration was toxic, fulfilling the guidance recommendation that significant cytotoxicity be observed. The Sponsor conducted both in vitro mammalian genotoxicity assays; that is, the in vitro assay for chromosome aberration and the in vitro assay for mammalian cell mutagenicity. Chromosome aberrations were evaluated in Chinese hamster ovary (CHO) cells. The initial RF assay used a 4-log concentration range and found significant toxicity at the highest concentrations with no viable cells. The definitive assay used three concentrations based on the RF assay. The exposure times were 4 and 24 hr in the absence of metabolic activation and 4 hr in the presence of metabolic activation. An increased cell endoreduplication was observed in cells treated at the highest two drug concentrations in the presence of metabolic activation. The biological significance of the finding was not known. This finding likely led the Sponsor to conduct the in vitro mammalian mutagenicity assay, the evaluation of HGRT mutations in CHO cells. The assay used concentrations over 3 logs incubated with drug for 20–24 hr in the absence of metabolic activation and 4 hr in the presence of metabolic activation. Apparently the dose RF assay was unable to reach a maximum concentration causing at least 80% cytotoxicity. The definitive study did reach cytotoxic concentrations in the presence, but

not the absence, of metabolic activation. The conclusion was that Celebrex was not mutagenic in this assay at the highest assayed concentrations. The *in vivo* micronucleus assay was conducted using male and female Sprague–Dawley rats. Five rats/sex were allocated to receive vehicle control, cyclophosphamide (positive control) or drug at 150, 300, or 450 mg/kg for 3 days. This study was conducted approximately 2 years after the 4-week repeated-dose toxicity studies. Rats were terminated 24 hr after the final dose and bone marrow was extracted from the tibia. Slides were evaluated for micronuclei. Celebrex was not clastogenic in this assay. Hence, the sum of the studies did not reveal a mutagenic potential for Celebrex.

The above studies make up the package of toxicity tests generally required for marketing approval. In the case of Celebrex, there was some additional toxicity testing conducted. These special toxicology tests are listed in [Table 12](#). The objective of the special testing of the drug was to evaluate antigenicity, skin sensitization, and the potential to cause local irritation to the skin or eyes. The rationale for carrying out these studies is not clear given that the drug is an oral product. However, the studies were conducted early in the development of the drug at about the same time that the 13-week repeat-dose toxicity studies were being conducted in rats; hence, they were not suggested by regulatory authorities late in the development of the drug. One explanation could be that the studies were conducted with the manufacturing personnel in mind, for the preparation of an material safety data sheet (MSDS), rather than the clinical population. That possibility is further evidenced by the conduct of skin sensitization and dermal and ocular irritation studies with starting chemical material for the synthesis of Celebrex. Acute toxicity testing in rats and an Ames assay were also conducted with the starting chemical material. Interestingly, the starting material was an extremely potent dermal sensitizer in guinea pigs and a local irritant, in contrast to the finished drug which was negative in all of the special toxicity studies.

Specific safety pharmacology studies were also conducted to support the marketing application. These studies are listed here but are not discussed in detail—the reader is encouraged to review the Summary Basis of Approval document. In mice, CNS effects were evaluated in single oral dose studies to monitor general activity and behavior, spontaneous locomotor activity, hexobarbital sleeping time, induced convulsions, and analgesia. In addition, a gastrointestinal transit study of the effect of a charcoal meal on the drug was conducted in mice. In rats, body temperature and renal effects were evaluated in animals given single oral doses. In dogs, drug effects (following administration of single oral dose) on respiratory and cardiovascular physiology were evaluated. Finally, in guinea pigs, the effect of drug concentrations on isolated ileum was evaluated to assess effect on autonomic nervous system and smooth muscle. These safety pharmacology studies encompassed the spectrum of examination of the major physiological

systems as outlined in the Japanese Guidelines for Safety Pharmacology evaluation (8).

In summary, the drug safety evaluation of Celebrex is a classic example of the package of studies required to support marketing approval of a drug with anticipated wide and potential long-term usage. The rat and the dog were the primary species in which repeat dose toxicity testing was conducted. Mice were used insofar as the carcinogenicity of the drug in the second species is required. A single dose monkey study was conducted, which is highly unusual given that the dog was an appropriate non-rodent species. The design of the studies and the comments by the FDA reviewer also reveal some interesting study components for the toxicologist to consider when planning a development program for his/her drug product.

5.2. Case study 2: Herceptin® (CPMP/1774/00, Trastuzumab)

Trastuzumab is a recombinant humanized monoclonal antibody (MAb) that binds to the extracellular domain of the human epidermal growth factor receptor 2 protein otherwise known as HER2. The MAb is an IgG1 kappa antibody that contains the human framework regions with the mouse complementary determining regions that bind to HER2. In the case study of Herceptin®, the European review document was used as the source of the studies conducted to support marketing approval in Europe. Roche is marketing authorization holder in Europe. The Scientific Discussion document for Herceptin can be found at the EMEA website at www.eudra.org/humandocs/humans/EPAR/Herceptin/Herceptin.htm. The Application for Marketing Authorization was submitted to EMEA on February 11, 1999 and the biologic was granted Marketing Authorization on May 25, 2000. The delays in approval were primarily related to manufacturing issues.

Trastuzumab, the active substance, is produced in a cell-based system, a CHO suspension culture. A significant sidebar of note is that an early development cell line was used to support non-clinical toxicity testing and Phase I and II clinical trials, while a later cell line was developed for production of the intended marketed product. The finished product, Herceptin, is administered as an initial loading dose of 4 mg/kg MAb over a 90-min infusion period, followed by weekly maintenance dose of 2 mg/kg given over a 30-min period. In the United States, the biotechnology-derived product is lyophilized and reconstituted with bacteriostatic water containing 1.1% benzyl alcohol as a preservative. The reconstituted solution is diluted in normal saline for infusion into the patient. In Europe, the use of a preservative is contrary to the Ph. Eur. requirements and the drug product is reconstituted with sterile water without preservative.

Herceptin is an interesting case study since it provides an overview of the extent of toxicity testing one might expect to conduct to support administration of a human biotechnology-derived protein. The list of toxicity

Table 13 Toxicity Studies Submitted to Support Marketing Authorization of Herceptin® in Europe

Primates	Other studies
Single dose i.v. toxicity study—rhesus monkeys	Single dose i.v. toxicity study—mice
4-week i.v. toxicity study—rhesus monkeys	Co-administration (cancer chemotherapeutics) PK/toxicity in rhesus monkeys
13-week i.v. toxicity study—cynomolgus monkeys	Local tolerance study—rabbitsI
26-week i.v. toxicity study—cynomolgus monkeys	Tissue cross-reactivity study—adult human and cynomolgus monkey tissues
Fertility and reproductive toxicity study—cynomolgus monkeys	In vitro mutagenicity in CHO
	In vitro chromosome aberration in CHO
	In vivo mouse bone marrow micronucleus assay

studies conducted in support of the marketing application for Herceptin is given in Table 13. The ICH guidance regarding the studies to support registration of a biotechnology drug product should be read in conjunction with this case study (ICH Topic S6, p. 20). As stated in the guidance, the toxicity studies should be conducted in the relevant species and, in some instances, this will result in the testing of the drug in only one species, as was the case for Herceptin. The selection of non-human primates for the toxicity studies was not, however, consistent with the action of the MAb in humans. In non-human primates, unlike humans, there is no overexpression of the p185^{HER2} protein and no production of shed antigen. In addition, non-human primates possess an uncharacterized protein on epithelial cells to which the humanized MAb binds. Nevertheless, monkeys were chosen as the most relevant species and the toxicity studies were mostly limited to monkeys.

With that said, although limited to a single species, the standard durations of repeated-dose toxicity studies were conducted. Hence, the package of studies (in terms of length of repeated dosing toxicity studies) parallels those studies submitted to support approval of the drug Celebrex. Thus, a single-dose toxicity study of trastuzumab was followed by studies of 4, 13, and 26 weeks. In Europe, prior to ICH Topic S4A, the standard length of a chronic repeated-dose toxicity study in non-rodents was 6 months (26 weeks) as opposed to the current ICH S4A guidance of 9 months. One interesting fact regarding the toxicity testing in monkeys was that the

program initially utilized the rhesus monkey while later studies utilized the cynomolgus monkey.

The single-dose toxicity of trastuzumab was evaluated in rhesus monkeys and in mice. The biologic was administered at 0, 4.7, 23.5, and 47 mg/kg by i.v. bolus to monkeys and at 0, 9.4, 47, and 94 mg/kg by i.v. bolus to mice. The review document notes that there were several different formulations evaluated. However, the details of these acute studies and the other toxicity studies are not provided to the level reported in the SBA document for Celebrex. Endpoints included clinical chemistry, determination of antibody formation, and gross and histopathological evaluations. The NOAEL was found to be the highest dose studied in either species; 47 and 94 mg/kg in rhesus monkeys and mice, respectively.

The repeated-dose toxicity studies are discussed as a single package of studies in the submission, and the study designs are not provided. Rhesus monkeys were used for the 4-week study, while cynomolgus monkeys were used for the 13- and 26-week studies. The reason for the switch to the cynomolgus monkeys is not known, but the reason may be that purpose-bred cynomolgus monkeys are more easily obtained than rhesus monkeys. No dose levels are provided in the review. Blood samples were collected for TK analyses, but no details of the sampling intervals are provided. There was minimal toxicity observed, with the single observation being injection site effects in the 4-week study. A rabbit local tolerance test, where trastuzumab was administered as a single bolus injection into the ear vein, revealed no local irritation. Neutralizing antibodies were detected in only one monkey, a single-dose female in the 26-week study. The overall incidence was 1/84 of monkeys treated with trastuzumab in repeated-dose studies.

There were no adverse toxicities in rhesus monkeys (in-life observations, clinical pathology only) when trastuzumab was co-administered with Taxol, adriamycin, or a Cytosan/adriamycin combination. However, co-administration with Taxol did result in a 2-fold reduction in clearance of trastuzumab; this was not seen with the other cytotoxic drugs.

Reproductive toxicity studies were conducted in cynomolgus monkeys with trastuzumab. This is somewhat unusual for a biologic that is not active in rats or rabbits, but likely reflects the intent to treat population, patients (women) with metastatic breast cancer whose tumors overexpress HER2. At dose levels of up to 25 times the weekly maintenance dose in women, there were no effects on reproductive function in females, no maternal toxicity or embryotoxicity, and no adverse peri-postnatal toxicities. These studies allowed for labeling of the biologic as a Pregnancy Category B. Placental transfer of the biologic was observed during the gestational period and this is noted on the labeling insert.

Trastuzumab was investigated for mutagenic potential in the ICH recommended battery of genetic toxicity tests. Again, for this class of biologic, genotoxicity is not an absolute requirement, but a review of recent

approvals of biologics by CBER shows an increasing trend to conduct these studies. An Ames bacterial mutagenicity assay, an in vitro evaluation of chromosome aberrations in human peripheral lymphocytes, and an in vivo mouse micronucleus assay (single i.v. dose of 29.5, 59, and 118 mg/kg) all gave negative mutagenicity results.

Tissue cross-reactivity studies were conducted with human and monkey tissues as requested in the FDA guidance for the testing of MAb (FDA, CBER, 1977) (25).

Finally, Herceptin does contain a boxed warning in the package label insert regarding the potential for cardiotoxicity; manifested as development of ventricular dysfunction and congestive heart failure. The single-dose toxicity study with trastuzumab and adriamycin discussed previously did not reveal any evidence of cardiac effects. Using a surrogate rat c-erb-B2 antibody, there was no enhancement of adriamycin-induced toxicity for trastuzumab. Further investigations in dogs were not conducted due to the lack of pharmacological activity in this species. Possible anthracycline models in monkeys were considered unsuitable based on lack of well-defined endpoints.

To sum, the toxicity studies conducted with Herceptin are illustrative of the toxicity testing of a biologic that is active only in man and in non-human primates. Reproductive toxicity and genotoxicity testing represent trends in study conduct to support marketing, although these are not absolute requirements. The use of a 26-week repeat-dose toxicity study to support chronic dosing is characteristic of the European guidance regarding chronic study duration prior to the ICH harmonization of the study duration at 9 months.

5.3. Case Study 3: Rituxan[®] (BLA 97-0260, Rituximab, IDEC-2BC8)

Rituximab is a chimeric (murine/human) IgG1K MAb directed against the CD20 antigen present on the surface of malignant, and normal, B lymphocytes. CD20 is present on the surface of greater than 90% of all B-cell non-Hodgkin lymphomas (NHL) (26). The IgG1 structure of the MAb is designed to possess effector function through binding of the Fc portion of the constant region of the MAb to the Fc γ receptors expressed on immune effector cells. Hence, the MAb binds specifically to CD20 on the malignant B-cell and linkage to the effector cell results in destruction of the tumor cell. The biologic is produced in CHO suspension culture and is provided as a concentrated liquid. The Sponsor, IDEC, submitted the Biologics License Application (BLA) for Rituxan, while Genentech submitted the additional manufacturing BLA 97-0244. The SBA can be found under the Freedom of Information act at www.fda.gov/cber/products/ritugen112697.htm.

Table 14 Toxicity Studies Submitted to Support Marketing Approval of Rituxan[®] in the United States

Primates	Other studies
Single dose, dose escalation i.v. toxicity study—cynomolgus monkeys; included TK	Tissue cross-reactivity study—adult human tissues
4-week and 8-week repeated dose i.v. toxicity study—cynomolgus monkeys; included TK	Single dose i.v. PK study—rats
Embryo-fetal development study—cynomolgus monkeys; including TK ^a	
Peri- and post-natal development study—cynomolgus monkeys ^a	

^aReproductive toxicity studies are being conducted post-approval for NHL as the Sponsor is looking to expand the clinical indication into non-life-threatening diseases. The embryo-fetal study has been completed and the peri- and post-natal development study is being planned as of March 2003.

The biologic was approved in November 1997 for the treatment of relapsed or refractory CD20+ B-cell NHL.

Rituxan is a good case study to include here, as an example of the limited scope of non-clinical toxicity testing that may be required for a drug (biologic) that is to be given for a short period of time and for the treatment of a life-threatening disease. The clinical course of treatment is 375 mg/m² given by i.v. infusion once weekly for 4 weeks. The list of toxicity studies conducted in support of the BLA for Rituxan is provided in Table 14.

Tissue cross-reactivity studies were conducted with human tissues as requested in the FDA guidance for the development/testing of monoclonal antibodies (25). It is not clear if studies were also conducted in cynomolgus monkey tissues, but there is no reference to monkey tissues in the SBA review document. It would be unusual if the Sponsor did not conduct such a study in monkey tissues, since the goal of tissue cross-reactivity studies, in addition to evaluating whether or not there is unexpected binding in human tissues, is to determine the comparability of binding between human and monkey tissues. Similar patterns of tissue cross-reactivity between humans and monkeys demonstrate that the monkey is a relevant laboratory animal species in which to perform toxicity testing.

The single-dose study in cynomolgus monkeys utilized a dose escalation study design. On monkey per sex per group was administered the drug intravenously at 10, 30, or 100 mg/kg and followed for 14 days of observation and blood sample collection (for TK and hematology purposes). As with a human Phase I clinical trial, the monkeys were

administered the low dose and followed for the observation period prior to initiating the subsequent higher dose to the next set of two monkeys. Blood sampling time points for TK were not provided in the review document, but the number of samples was sufficient to allow the Sponsor to define one- and two-compartment kinetic models. Transient decrements in the numbers of lymphocytes were observed in all dose groups.

The repeat-dose toxicity study consisted of four groups of cynomolgus monkeys. Groups 1 and 2 were administered vehicle ($n = 1/\text{sex}/\text{group}$) while Groups 3 and 4 were given 20 mg/kg Rituximab ($n = 3/\text{sex}/\text{group}$). Groups 1 and 3 were dosed weekly for 4 weeks and Groups 2 and 4 were dosed weekly for 8 weeks. Monkeys in all groups were terminated 2 weeks after the last dose. Clinical pathology assessments were made on all animals at time points during the study, and a necropsy was conducted on all animals at termination. There were decrements in the numbers of B-cells in the MAb-treatment groups and histopathological evidence for depletion of B-cells in spleens and lymph nodes. Somewhat unusual in this study was the fact that animals were not terminated until 2 weeks after final dosing, allowing some recovery from compound effects. It is more common in the design of a repeat-dose study to include subgroups, one terminated following final dosing and the other allowed a recovery period.

The repeat-dose toxicity study included an assessment of TK, although time points were not provided. Given the long half-life relative to the dosing interval (that is, the MAb may not have been eliminated significantly from the body before the next subsequent dose of MAb was administered), there was likely not a collection of full profiles but rather an assessment of peaks and troughs following dosing. While repeat-dose studies often do not collect full profiles during the first doses of the compound (since the dose interval is too frequent to allow adequate determination of pharmacokinetic parameters such as half-life), a study will often include sample collection at numerous time points following the final dose to determine pharmacokinetic parameters. In this repeat-dose toxicity study, such data collection and interpretation would have been hindered by the fact that anti-MAb antibodies were observed in all monkeys after repeated doses of Rituximab.

The pharmacokinetics of different lots and methods of manufacturing of the MAb were also studied in Sprague-Dawley rats and cynomolgus monkeys to demonstrate bioequivalence; however, details regarding study design were not provided in the SBA review document.

Rituximab was not evaluated in genotoxicity or carcinogenicity testing. Such non-clinical testing is not an absolute requirement for this class of drug (biologic) intended for a life-threatening clinical indication. The initial approval of Rituxan also did not include any reproductive toxicity testing, and the current approved labeling is Pregnancy Category C, noting that animal reproductive toxicity studies have not been conducted.

While the initial filing and approval of Rituxan for the treatment of CD20+ B-cell NHL was based on the limited toxicity testing outlined in Table 14, this limited package of toxicity studies would likely not support approval of the MAb for non-life-threatening disease indications. In looking to expand the clinical indication for Rituxan into immune disorders (for example, rheumatoid arthritis), the Sponsor has completed an embryo–fetal development study in cynomolgus monkeys and is presently defining a peri- and post-natal development study in cynomolgus monkeys (27).

In the embryo–fetal development study, 48 female cynomolgus monkeys were allocated 12/group to receive vehicle control or Rituximab at 20, 50, or 100 mg/kg weekly from gestational day 20 to gestational day 50; loading doses of 0, 15, 37.5, and 75 mg/kg, respectively, were administered on gestational days 20, 21, and 22 to provide consistent exposure. The study parameters included clinical pathology determinations, and collection of blood samples for MAb and anti-MAb antibodies. Monkeys underwent cesarian section on gestational Day 100. All fetuses were examined externally and visceral and skeletal examinations were conducted. The spleen and lymph nodes were also examined histopathologically and immunohistochemically. Maternal and fetal systemic exposures to rituximab were confirmed at C-section. There were no adverse treatment-related effects on embryo–fetal development.

The abbreviated non-clinical toxicity package of studies to support marketing approval of Rituxan is an example of what might be considered the minimal testing platform that one will likely encounter. The limited scope is based on the clinical indication for a life-threatening disease and the limited duration of treatment. The case study also illustrates how additional toxicity studies may be required as the clinical indication is expanded.

5.4. Case study 4: Remicade® (BLA 98–0012, Infliximab)

Infliximab is a chimeric (murine/human) MAb containing the murine variable region amino acid sequence. Approximately 30% of sequence is murine, with the remaining 70% of the antibody corresponding to human IgG1K. The MAb binds specifically to human tumor necrosis factor alpha (TNF α). TNF α is a pro-inflammatory cytokine and elevated levels of the cytokine are thought to play a role in a number of immune disorders. The MAb binds to both the soluble and transmembrane forms of TNF α thereby inhibiting the binding of TNF α to its receptor. Binding to the soluble form results in an amelioration of the induction of pro-inflammatory cytokines and migration of inflammatory cells to areas of inflammation. Like Rituxan, infliximab is an IgG1 isotype intended to have effector function, and binding of the MAb to transmembrane TNF α leads to cell lysis by effector cells or complement.

The first approved indication for Remicade was for Crohn's disease and this case study is primarily based upon the Summary Basis of Approval

document for this indication found at www.fda.gov/cber/products/inflcen082498.htm. The Sponsor of this BLA was Centocor. The BLA was received at CBER on December 30, 1997, and the Pharmacology review was completed on May 12, 1998. The intended treatment regimen for Crohn's disease is three intravenous administrations of 5 mg/kg (an initial dose and at 2 and 6 weeks). One should be cognizant of this short duration of treatment when reviewing the studies submitted in the Summary Basis of Approval.

The initial indication for Crohn's disease was subsequently expanded to include rheumatoid arthritis, in which 3 mg/kg Remicade is administered (intravenous infusion) initially, at 2 and 6 weeks, and then every 8 weeks thereafter (25). The labeling also provides for dosing of up to 10 mg/kg administered every 4 weeks. The Summary Basis of Approval (USA) for this indication is not available, however, additional data can be found in the European Scientific Discussion document for Marketing Authorization in Europe for Remicade (CPMP/1901/99); the website is www.eudra.org/humandocs/Humans/EPAR/Remicade/Remicade.htm. In addition, the Physician's Desk Reference (PDR) is especially useful as the animal toxicity labeling has changed several times during the span of 2000–2003 (28–30). The use of both FDA and EMEA documents in this case study provides a sense of the toxicity information required when moving from the limited treatment of Crohn's disease to the more prolonged treatment of rheumatoid arthritis.

Two tissue cross-reactivity studies were conducted with human tissues as requested in the FDA guidance for the development/testing of monoclonal antibodies (25). Cross-reactivity was observed in cells and tissues known to express TNF α ; no unexpected cross-reactivity was observed. The ability of infliximab to neutralize TNF α activity in vitro was the assay used to investigate the species cross-reactivity of the biologic. No inhibition of activity was observed using TNF α from baboon, rhesus, and cynomolgus monkeys, pig-tailed macaque, marmoset, tamarin, pig, rabbit, rat, or mouse. The potency in the dog was five orders of magnitude less than that observed with human and chimpanzee TNF α . The cross-reactivity with chimpanzee TNF α was expected since the protein sequence of the chimpanzee cDNA was identical to that of humans.

The lack of a relevant species for infliximab and the fact that the treatment regimen (initially solely for Crohn's disease) consisted of three total infusions provides the rationale for a very atypical non-clinical testing program to support marketing authorization of Remicade (Table 15). Single- and repeat-dose toxicity studies were conducted in chimpanzees and reproductive toxicity studies were conducted in mice with a murine analog of infliximab. Two single-dose pilot studies were conducted in dogs since there was some reactivity with dog TNF α in vitro, albeit orders of magnitude less than seen in humans. However, an immediate hypersensitivity reaction was observed in association with increased plasma histamine levels and, consequently, the dog was not considered a practical species in which to

Table 15 Toxicity Studies Submitted to Support Marketing Approval of Remicade® for Crohn's Disease

Chimpanzees	Mice (with murine analog cV1q MAb)	Other studies
Single-dose i.v. toxicity study	RF embryo–fetal development study—cV1q MAb	In vitro inhibition of TNF α activity in multiple animal species—rhesus and cynomolgus monkeys, marmoset, tamarin, pig, rabbit, rat, mouse tissues
Single- and repeated-dose study (up to 5-dose) i.v. toxicity study	Embryo–fetal development study—cV1q Mab	Tissue cross-reactivity study—adult human tissues
3-day i.v. toxicity study	6-month toxicity/tumorigenicity study—cV1q MAb ^a	Single dose i.v. toxicity study—rats (two studies)
		7-day i.v. toxicity studies—rats (three studies)
		Single-dose i.v. and IM studies—rabbits
	Tumorigenicity studies in mice deficient in TNF α ^a	Ames bacterial mutagenicity assay
		In vitro chromosome aberration assay in human lymphocytes
		In vivo mouse micronucleus assay

^aThese studies were not submitted in the initial BLA, but are found in the later EMEA Scientific Discussion document to support Marketing Authorization.

evaluate the toxicity of the biologic. Toxicity studies were also conducted in rats, but the FDA reviewer noted that these studies have little relevance to the safety evaluation and served only to evaluate non-specific toxicities.

Before discussing these non-clinical toxicity studies, it is of interest to note that infliximab is not unique as a MAb that is cross-reactive only in humans and chimpanzee. Keliximab is a PrimatizedTM anti-CD4 MAb that was in development for the treatment of autoimmune disorders. Like infliximab, this MAb recognized only human and chimpanzee CD, and not CD4 from other species including rhesus and cynomolgus monkeys. Consequently, like infliximab, non-clinical studies with keliximab were conducted in chimpanzees (31). However, rather than utilize a murine analog of the MAb, non-clinical studies with keliximab used a different approach, the administration of the human MAb to transgenic mice expressing human

CD4 (32). The reader is referred to these papers to note the similarity in the non-clinical development programs.

In the single-dose study, infliximab was administered to one male and one female chimpanzee (i.v. dose 30 mg/kg). This study was conducted to support the use of the biologic in combination with Centoxin[®] (an MAb to an endotoxin protein from Gram-negative bacteria). Both chimpanzees were monitored for 14 days, with collection of ECGs, ophthalmic examinations, clinical pathology, and blood samples (to determine MAb concentrations) and anti-infliximab determinations. Infliximab was well tolerated in this study.

In the single-dose/multiple-dose study, one male/group was administered a single dose of vehicle or 30 mg/kg, one male and one female received 30 mg/kg for three consecutive days, and two males and one female received 15 mg/kg for either four or five consecutive days. Two additional females were administered vehicle for either three or five consecutive days. All chimpanzees were monitored for 2 weeks with the same collection of parameters as described for the single-dose study. In this study (as in all of the chimpanzee studies) animals were anesthetized for infusion of the test article. However, in this particular case, cumulative doses of ketamine were included since sedation and respiratory depression were observed in one animal treated with vehicle, and 15 and 30 mg/kg dose groups. The anesthesia protocol was altered and an additional two animals were administered 30 mg/kg for three consecutive days. Other than the effects related to anesthesia, there were no adverse effects associated with infliximab infusion.

In the final chimpanzee toxicity study, one sex/group received vehicle or 30 mg/kg infliximab by i.v. infusion (1–2 hr) for three consecutive days. In this study, animals were monitored for 6 weeks. Observations included ECGs prior to, during and immediately after the first and last dose, on Day 4, and at termination. Ophthalmologic examination was performed prior to each dose and at termination. Blood samples for clinical pathology, serum infliximab concentrations, and anti-MAB antibodies were collected prior to the first two doses, on Day 4 and weekly thereafter. As with the other studies, there were no adverse effects of infliximab in this study.

In summary, infliximab was well tolerated at doses of 30 mg/kg for three consecutive days and 15 mg/kg for five consecutive days with no treatment-related toxicities. Systemic exposures were demonstrated in all treatment groups. Anti-infliximab antibody determinations were problematic. Due to the long elimination half-life of the compound, Infliximab was still present in the blood (the presence of the MAB would interfere with the ELISA assay by binding any anti-MAB antibodies that might be present in the blood).

Local tolerance testing was carried out in rabbits to evaluate non-specific irritant effects. An acute i.v. study was conducted in which groups of 16 male New Zealand White rabbits were allocated to receive 3 hr

infusions of infliximab, human serum albumin, or saline for injection. Additional groups received similar treatment subcutaneously to evaluate potential extravasation during infusion. The i.v. infusions were well tolerated, although subcutaneous administration of infliximab did cause slightly greater irritation relative to the other treatments. In the acute intramuscular study, seven NZW male rabbits were assigned to receive infliximab, human serum albumin, or cefoxitin sodium (Mefoxin[®]) at separate sites (the same animal received all treatments at separate sites thereby serving as its own control). Injection sites were scored daily for 3 days. Infliximab produced less irritation than Mefoxin, a drug approved for intramuscular injection.

As discussed previously, a relevant animal model for an evaluation of the reproductive toxicity of infliximab was not available because infliximab was not reactive with TNF α from rats or rabbits. Nonetheless, it was important for the Sponsor to obtain a Pregnancy Category B labeling for Remicade, and the Sponsor pursued this by conducting embryo–fetal studies of a murine analog, cV1q, in mice. This murine MAb cV1q was validated for specificity for murine TNF α *in vitro*, and the pharmacological activity was validated *in vivo* in a transgenic murine colitis model.

An exploratory embryo–fetal study was first conducted in which groups of 16 mated female mice were allocated to receive an i.v. bolus of 0, 10, 20, or 40 mg/kg cV1q on gestation day 6 (gestational day 6); the 40 mg/kg dose was determined to be the therapeutic dose in mice in a murine model of colitis. Blood samples were collected at 24 hr, 4, 7, and 12 days after dosing on gestational day 6 from 4/group at each time point. The blood collection was terminal. A C-section and gross necropsy were performed and fetal endpoints (e.g., numbers of corpora lutea, implantation sites, viable/non-viable fetuses, fetus weight) were evaluated.

The definitive embryo–fetal study was conducted with groups of 33 mated female mice allocated to receive an i.v. bolus of 0, 10, or 40 mg/kg cV1q. Since blood levels of cV1q in the exploratory study were not maintained out to gestational day 18, mice were treated on gestational day 6 and again on gestational day 12. Due to a shortage of cV1q antibody, only 22 and 23 mice in the 10 and 40 mg/kg groups, respectively, were dosed on gestational day 12. On gestational day 14, 8 mice/group underwent cesarean section and blood samples were collected for maternal and fetal MAb concentrations and a gross necropsy was conducted for determination of fetal parameters. Remaining mice were terminated on gestational day 17 with collection of maternal blood samples. Fetuses were evaluated for gross external alterations and one-half of the fetuses were examined for visceral alterations, and the other half for skeletal alterations. Administration of cV1q was not associated with any maternal or developmental toxicity. Maternal and fetal blood concentrations of cV1q were observed, indicating fetal exposure throughout organogenesis. The murine analog cV1q had the same specificity, pharmacological activity, and pharmacokinetic profile as

infliximab. Consequently, the reproductive toxicity studies in mice with the murine analog, with the lack of any adverse reproductive toxicity, were determined by the FDA reviewers to be relevant for the pregnancy class labeling and inclusion in the Remicade package insert.

A 6-month study of the murine analog cV1q was submitted to the EMEA in 2001 (see also the later text pertaining to carcinogenicity of Remicade). A likely presumption is that this study was required to support the extended duration of treatment in rheumatoid arthritis. This is revealing in that one might expect the Sponsor to argue that the plethora of human safety data generated from the approved use of the product in Crohn's disease would be of more relevance to the assessment of safety for the extended treatment for rheumatoid arthritis than would chronic animal data. Either the regulatory authorities asked for this study or the Sponsor acted proactively in conducting the study and providing the data. Mice received 25 weekly doses of 0, 10, or 40 mg/kg by i.v. infusion (study protocol elements are lacking). The study did not find any treatment-related mortality, clinical signs, or histopathological findings, but there was a dose-dependent increased incidence of bilateral crystalline deposits in the lens capsules of male mice. The relevance of this finding to humans is not known.

Genotoxicity assays are not required for monoclonal antibodies, but infliximab is another example of the studies nevertheless being conducted. Infliximab was evaluated in the Ames assay, in an in vitro chromosome aberration assay with human lymphocytes, and an in vivo mouse micronucleus test. Infliximab was non-genotoxic in all of these assays.

Carcinogenicity bioassays also are not typically required for monoclonal antibodies, yet this has been an area of interest for Remicade and the progression of the package labeling in the PDR is notable. The Remicade package insert in the 2000 PDR for Crohn's disease states that long-term animal studies to evaluate carcinogenicity have not been conducted (29). However, the labeling does make reference in the Precautions and Adverse Reactions to the patient population being at risk for the development of lymphoma and the unknown impact of Remicade on the development of malignancy. In the Remicade package labeling in the 2002 PDR for the treatment of both Crohn's disease and rheumatoid arthritis, the Precautions include the following statement "Tumorigenicity studies in mice deficient in TNF α demonstrated no increase in tumors when challenged with known tumor initiators and/or promoters." (30). Interestingly enough, this labeling statement may have come from the submission of literature data and not specific studies conducted by the Sponsor. This conclusion is based on the EMEA Scientific Discussion, which mentions that information from studies with TNF α knock-out mice and mice treated with murine anti-TNF α does not provide evidence of an increased risk of tumor development. There is no reference to a specific study with the murine cV1q MAb. Regardless, it is

interesting that the Sponsor was able to obtain on the package labeling this specific reference to lack of carcinogenicity in laboratory animals deficient in TNF α . The Remicade labeling pertaining to carcinogenicity was again revised in the 2003 PDR (28). This labeling would indicate that the 6-month chronic study of cV1q in mice discussed previously was actually a tumorigenicity study; although tumorigenicity is not mentioned in the EMEA Scientific Discussion document comments on this 6-month study. As the dose levels and duration are the same, this is likely one and the same study. The Remicade labeling in the 2003 PDR has dropped the reference to the lack of tumorigenicity in TNF α -deficient mice provided in the 2002 PDR, and stated that a 6-month repeated-dose study with cV1q anti-mouse TNF α found no indication of tumorigenicity in mice.

In summary, the lack of cross-reactivity and a relevant animal species, the expanded clinical population, and the extended clinical dosing regimen provide for a fascinating Case Study of Remicade. Remicade was first approved for use in patients with Crohn's disease in which a total of three doses of the biologic were to be administered. As infliximab was found to be cross-reactive only with TNF α in chimpanzee, an endangered species, the regulatory authorities did not have serious issues with the lack of repeated-dose administration non-clinical toxicity data to support the short duration of clinical exposures. Nevertheless, the Sponsor was concerned with the Pregnancy category labeling and developed a murine surrogate for use in reproductive toxicity testing in mice. The negative data generated in these studies allowed the Sponsor to use a Pregnancy Category B in the labeling package insert. It is interesting to note that in moving Remicade forward in the treatment of rheumatoid arthritis, the availability of safety data for the biologic in thousands of Crohn's disease patients did not preclude the Sponsor from conducting a chronic toxicity/tumorigenicity study of the murine analog. The submission of novel carcinogenicity studies not involving administration of the biologic in question to support a labeling statement is also a remarkable component of this Case Study, as is the progression in the PDR (consecutive years) of the studies included in the carcinogenicity section of the package labeling.

6. CONCLUSIONS

Drug development is a dynamic, ever-changing process that is not easily compartmentalized into a checklist of non-clinical toxicity studies that should be conducted as one moves towards marketing approval. Despite the challenges in study design, the questions to be addressed in these studies generally remain the same. Characterization of target organs, dose-response relationships and correlative systemic exposures, and reversibility of any observed effects are all major issues that must be addressed in laboratory animals prior to administration of the drug in the first Phase I clinical trials.

The safety of the study drug continues to be investigated by the toxicologist during the conduct of Phase II and Phase III clinical trials. Non-clinical toxicity studies of greater duration and specialized toxicity studies investigating the potential of the drug to affect, for example, fertility and carcinogenicity, are conducted to provide additional support for the safety of the drug as it is administered in clinical trials utilizing more prolonged exposures and/or greater numbers of subjects. Subsequent to the completion of these non-clinical toxicity studies, the toxicologist will complete an overall assessment of the safety of the drug based on the non-clinical toxicity data, and will provide the pertinent toxicity data to be included in the package insert of the product.

The design of the overall non-clinical package of toxicity studies and the conduct of the individual studies is best thought of as an art form rather than a static exercise in standardized studies. The toxicologist must tailor each study to fit the intended clinical population and the properties of the drug or biologic in question. The case studies provided in this chapter provide excellent examples of the differences in the package of studies submitted for approval. In this context, a fascinating range of non-clinical studies and study packages was explored, from Celebrex[®], a drug with activity in a wide range of animal species intended to treat non-life-threatening indications of rheumatoid arthritis and/or acute and chronic pain in a potentially large patient population with intermittent drug exposures over a lifetime, to Rituxan, a biologic with restricted activity in animal species intended to treat patients with life-threatening B-cell NHL. The toxicity studies, individually and in the sum package of studies, have posed challenges for the toxicologist, whether it be in study design, conduct of the in-life segment of the study, interpretation of the study results, and/or applicability to the clinical population.

In the past, regulatory authorities from the different regions of the world have added their different views regarding both the overall package of studies required to support clinical trials and the protocol elements of the studies themselves, further complicating the already dynamic nature inherent in the design and conduct of the package of non-clinical studies. To confront these differences in viewpoints, the formation of ICH has been an invaluable endeavor to provide guidances in the safety assessment of a drug, as well as in the areas of quality and clinical trials. The application of these guidances has eliminated redundancies across the three major regions comprised of the United States, Europe, and Japan. Equally important, ICH has addressed the science behind the toxicity studies and has opened a constructive dialogue among regulatory authorities and the pharmaceutical industry. The work accomplished by ICH has been significant and represents important contributions from both sides. Just as drug development is an ever-changing process, so too will ICH continue to evolve

and address areas of concern, all the while with an eye to the goal of improving human health.

APPENDIX

WEBSITES OF INTEREST

ICH: <http://www.ich.org/>

EC: <http://pharmacos.eudra.org/>

EMA: <http://www.ema.eu.int/>

EFPIA: <http://www.efpia.org/>

MHLW: <http://www.mhlw.go.jp/english/>

JPMA: <http://www.jpma.or.jp/12english/>

FDA: <http://www.fda.gov/>

FDA CDER Guidance Documents: <http://www.fda.gov/cder/guidance/index.htm>

FDA CBER Guidance Documents: <http://www.fda.gov/cber/guidelines.htm>

FDA Redbook 2000: <http://www.cfsan.fda.gov/~redbook/red-toca.html>

Federal Register Online: http://www.access.gpo.gov/su_docs/aces/aces140.html

PhRMA: <http://www.phrma.org/>

IFPMA: <http://www.ifpma.org/>

WHO: <http://www.who.int/en/>

EFTA: <http://secretariat.efta.int/>

TPD: <http://www.hc-sc.gc.ca/hpb-dgps/therapeut/htmleng/index.html>

Medicines Control Agency (MCA) of the United Kingdom
Department of Health: <http://www.mca.gov.uk/>

Therapeutic Goods Administration (TGA) of the Australian
Department of Health and Ageing: <http://www.health.gov.au/tga/index.htm>

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12. ICH Topic S5B Document “Maintenance to the ICH Guideline on Toxicity to Male Fertility.” Step 4 reached November 29, 1995 and amended on November 9, 2000.
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22. ICH Topic S3B Document "Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies." Step 4 reached October 27, 1994.
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Application of Pathology in Safety Assessment

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1. INTRODUCTION

1.1. Positioning of Pathology

Pathology contributes two main aspects to toxicological studies, namely results from

- Clinical pathology for detection of *biochemical toxicity* and from
- Postmortem examinations for detection of *structural toxicity*

Pathological investigations are a cornerstone for the assessment of the general toxicity of a compound and postmortem examinations are *the* endpoint in life-time rodent bioassays (carcinogenicity studies) (1). Pathology has also become increasingly important for research support (2–5) and histopathological examinations are performed for the clarification of certain macroscopic findings in reproductive toxicology studies (not covered in this chapter) (6).

Pathology is a holistic investigative tool, which allows conclusions to be drawn as to the overall integrity or sickness of an individual. In-life observations and clinical pathology findings in blood, serum, and urine are important elements of a toxicity study. However, they are rarely the basis for crucial decisions such as discontinuation of development of a compound in

the absence of corresponding pathological findings. Exceptions include electrocardiographic findings (in particular Q-T prolongation) and seizures. Both of these changes are related to *functional toxicity*, where pathological alterations are minimal or not detectable with conventional techniques.

Pathologists often act as study directors of toxicity evaluations and particularly of life-time bioassays. In cases where his/her duties are limited to clinical and/or postmortem pathology, close interaction between study director and pathologist is a valuable necessity. The pathologist should be involved from the planning to the reporting of the study and must be ready to answer questions posed by regulatory authorities, especially, but not only in case of carcinogenicity studies (see also Table 1) (7).

Table 1 Involvement of the Study Pathologist During Different Phases of a Toxicity/Carcinogenicity Study

Phase	Involvement of pathologist
Study planning	Contributes to design of the study including dose selection and parameters to be investigated, particularly in view of previous pathological findings
Study conduct	Works closely with study director. Needs to see important in-life observations
Necropsy (macroscopic inspection, organ weights, sampling)	Supervises necropsy, generally conducted by experienced technicians
Histotechnology	May supervise organ trimming, generally conducted by experienced technicians
Histopathological evaluation	Conducts evaluation and records findings Correlates pathological findings in different organs and with in-life observations including clinical pathology results
Report writing	Writes pathology section of report and contributes to the overall assessment of the findings
Preparation of registration documents	Reviews the toxicology-related part of the registration documents, in particular, the final toxicology summary and assessment
Answering of regulatory queries	Contributes to and reviews the answers to regulatory queries related to general toxicology

Shown is the minimal involvement. The pathologist may also act as study director, final report writer, and responsible scientist for writing the toxicology-related registration documents (2,4,5,38).

This book chapter is written mainly for nonpathologists, to provide them with an overview of procedures used in postmortem toxicologic pathology, to increase their understanding of the terminology used by pathologists and to ease their collaboration with pathologists during the conduct and evaluation of toxicity studies.

1.2 Pathology Methods

1.2.1. Clinical Pathology

Clinical pathology investigations are related to blood cells (hematology), clinical chemistry analysis of blood (e.g., pattern and amount of enzymes leaking from organs or tissues) and urine (urinalysis), and investigation of the coagulation of blood (8). A selection of important clinical pathology parameters is provided in [Tables 2A](#) and [B](#).

Investigation of clinical pathology parameters are undertaken as follows:

- Nonrodents—all animals and all doses: pretest, during treatment (see below), at the end of treatment, and—if applicable—at the end of the treatment-free recovery period
- Rodents—specialized investigations can be limited to a subset of animals at each dose level and control groups or to top dose and control groups during treatment (see below), at the end of treatment, and—if applicable—at the end of the treatment-free recovery period.

Investigation during treatment means sampling at appropriately spaced intervals (e.g., sample after 3 months for 6-month chronic toxicity studies).

For details, the reader is referred to specialized textbooks and articles (9–11).

1.2.2. Postmortem Investigations

Postmortem investigations start with a macroscopic inspection of the carcass and—upon dissection—of the internal organs and tissues by eye. With exceptions (e.g., acute toxicity studies) macroscopic inspection is followed by microscopic evaluation of samples of organs, tissues, cells, and cellular components in tissue sections or smears by means of a light or electron microscope. Microscopic inspections need histological processing of organ/tissue sections (for details see [Sec. 2](#) below).

For answering specific questions, microscopic investigations can be complemented by special methods such as:

- *Morphometry* to count numbers (e.g., cells per surface area) or dimensions such as diameters and volumes (12–15). Digital image
- (text continued on p. 429)*

Table 2A Clinical Pathology

Code	Parameter	Method of determination (example)	Unit ^a
<i>Hematology^b</i>			
Hb	Hemoglobin concentration	Spectrophotometric measurement of cyanmethemoglobin	g/dL or mmol/L
RBC	Red blood cell count	Flow cytometry	$10^{12}/L$
PCV	Packed-cell volume	<i>Calculated</i> (PCV=MCV $\times$ RBC) $\times$ correction factor depending on units used or <i>measured</i> by sedimentation	%
RETA	Reticulocytes	Supravital staining with oxazin 750, assessed by flow cytometry	% of RBC
MCV	Mean corpuscular volume	Flow cytometry	fL
MCH	Mean corpuscular hemoglobin	MCH = (Hb/RBC) $\times$ correction factor depending on units used	pg or fmol
MCHC	Mean corpuscular hemoglobin concentration	MCHC = Hb/(PCV $\times$ 100)	g/dL or mmol/L

PLAT	Platelets	Flow cytometry	$10^9/\text{L}$
MPV	Mean platelet volume	Flow cytometry	fL
PT	Prothrombin time	Fibrin formulation using rabbit brain thromboplastin to activate coagulation cascade	sec
APTT	Activated partial thromboplastin time	Fibrin formulation using micronized silica to activate coagulation cascade	sec
WBC	White blood cell count	Flow cytometry	$10^9/\text{L}$
N	Neutrophils	<i>Manual differentiation:</i>	% of WBC
L	Lymphocytes	Visual appraisal of a blood smear using a Romanowski-type stain	
M	Monocytes	<i>Automated differentiation:</i>	
E	Eosinophils	Cytochemical staining with a peroxidase stain and detection of light scatter using a photo-optical method.	
B	Basophils	Basophils enumerated using differential lysis and laser light scattering	

(Continued)

Table 2A Clinical Pathology (*Continued*)

Code	Parameter	Method of determination (examples)	Increased (examples)	Decreased (examples)
<i>Clinical blood chemistry^c</i>				
AM	Amylase ^d	Enzyme activity measured photometrically as NAD–NADH concentrations	Pancreatic diseases	Usually not clinically significant
γ -GT	γ -Glutamyltransferase ^d	Quantitative enzyme detection method	Hepatic disease Generally parallels ALK PHOS in hepatobiliary diseases Cholestasis Hepatic fibrosis	Not clinically significant
AST	Aspartate aminotransferase	Optimized UV method using L-alanine and α -oxoglutarate as primary substrates	Hepatitis Hepatic necrosis Skeletal muscle damage Myocardial necrosis Acute pancreatitis Hemolysis (artifact)	Not clinically significant
ALT	Alanine aminotransferase	Optimized UV method using L-alanine and α -oxoglutarate as primary substrates	Hepatitis Hepatic necrosis Cholestasis Hepatic neoplasms	Not clinically significant

ALK PHOS	Alkaline phosphatase	Colorimetric method using <i>p</i> -nitrophenyl phosphate as substrate	Steroids Cholestatic disorders Acute pancreatitis Neoplasms of or in bone Diabetes mellitus Certain drugs (e.g., anticonvulsants)	Not clinically significant
CK	Creatinine kinase ^d	Enzyme activity measured photometrically as NAD–NADH concentrations	Seizures Myocardial necrosis Intramuscular injections Hemolysis (artifact)	Not clinically significant Lack of refrigeration of serum
GLDH	Glutamic dehydrogenase ^d	Quantitative enzyme detection method based on NADH concentrations	Hepatic necrosis/inflammation Bile-duct obstruction	Not clinically significant
Na	Sodium	Ion-selective electrode	Hemolysis (except dogs) Adrenocortical hyperfunction	Hemolysis (in dogs) Excessive loss (e.g., hypoadrenocorticism, vomiting, diarrhea, renal failure)
K	Potassium	Ion-selective electrode	Hypoadrenocorticism (Addison's) disease Decreased excretion (e.g., renal failure) Improper sample collection and handling (hemolysis in some species)	Hyperaldosteronism

(Continued)

Table 2A Clinical Pathology (*Continued*)

Code	Parameter	Method of determination (examples)	Increased (examples)	Decreased (examples)
Cl	Chloride	Ion-selective electrode	All causes of increased sodium	All causes of decreased sodium
Ca	Calcium	Colorimetric method using <i>o</i> -cresolphthalein complexone in alkaline solution	Primary hyperparathyroidism (e.g., because of parathyroid adenoma or carcinoma)	Malabsorption Pancreatic necrosis Renal secondary hyperparathyroidism Hemolysis (artifact)
IN PHOS	Inorganic phosphorous	Colorimetric method based upon ammonium molybdate in acid conditions	Decreased renal clearance (e.g., renal disease/failure, hypoparathyroidism) Hemolysis or aging RBCs in sample	Malabsorption Hyperparathyroidism (primary or secondary)
Mg	Magnesium	Colorimetric method with Calmagit	Renal failure Adrenocortical insufficiency	Decreased absorption, reduced feeding Renal loss (e.g., increased diuresis)
UREA	Urea	UV method using a coupled urease procedure	Pre-renal azotemia (e.g., dehydration, hypoadrenocorticism) Renal azotemia (kidney failure) Post-renal azotemia (e.g., obstruction)	Decreased synthesis (e.g., severe hepatic disease)

T BILI	Total bilirubin	A colorimetric method. Indirect bilirubin is liberated by detergent. Total bilirubin is coupled with a diazonium compound to give corresponding azobilirubin	Hemolysis Hepatic disease (e.g., cholestasis, hepatocellular injury/failure) Fasting	Exposure of serum to sunlight Decreased RBC production
CREAT	Creatinine	Colorimetric method	Decreased renal perfusion (e.g., dehydration, hypoadrenocorticism, heart failure) Renal azotemia (kidney failure) Post-renal azotemia (e.g., obstruction)	Not clinically significant
T PROT	Total protein plus generally electrophoretic investigation of proteins	Colorimetric method based on Biuret reaction	Monoclonal gammopathies (e.g., multiple myeloma) Polyclonal gammopathy (e.g., infections, hepatic disease) Lipemia (artifact) Hemolysis (artifact)	Chronic hepatic disease Malabsorption Nephrosis Glomerulonephritis
ALBUMIN (A)	Albumin	Colorimetric method based on the BCG reaction	Dehydration Lipemia (artifact)	Increased loss (e.g., nephritic syndrome, glomerulonephritis) Vasculitis Decreased production (e.g., chronic hepatic disease)

(Continued)

Table 2A Clinical Pathology (*Continued*)

Code	Parameter	Method of determination (examples)	Increased (examples)	Decreased (examples)
GLOBULIN (G)	Globulin	Globulin = total protein – albumin	Polyclonal gammopathy (e.g., infections or parasites) Monoclonal gammopathy (e.g., myeloma) Hepatopathy	Decreased production (e.g., hepatic disease, immunodeficiency)
A:G RATIO	Albumin/globulin ratio	AG ratio = albumin/(total protein – albumin)	Hyperalbuminemia with normal or low globulins	Hyperglobulinemia with normal or low albumin
TOT CHOL	Total cholesterol	Enzymatic method using cholesterol oxidase/esterase	Hypothyroidism Increased fat mobilization (e.g., diabetes mellitus, hyperadrenocorticism), pancreatitis, cholestatic diseases, nephrotic syndrome Recently fed; high-fat diet (usually only mild increase)	Decreased uptake (e.g., low-fat diet, malabsorption) Decreased production (e.g., advanced hepatic disease) Increased loss or catabolism (e.g., protein-losing enteropathy, hyperthyroidism)

TG	Triglycerides	Colorimetric method using peroxidase	Hepatic disorders including biliary obstruction Hypothyroidism (see also under TOT CHOL) Transitory (stress) Diabetes mellitus, pancreatitis, hyperadrenocorticism, epinephrine, pituitary neoplasm, pheochromocytoma, hyperthyroidism	Estrogens Spironolactone Other reasons (see also under TOT CHOL) Adrenal insufficiency Hyperinsulinism (insulinoma) Hepatic disease
GLUC	Glucose	UV method using a coupled hexokinase procedure		
<i>Hormones:</i> Peptide hormones	Luteinizing hormone (LH) Follicle stimulating hormone (FSH) Thyroid stimulating hormone (TSH) Adrenocorticotrophic hormone (ACTH) Growth hormone (GH), etc.	Radioimmunoassay (RIA)	Hypertrophy, hyperplasia, and neoplasia of organ, where hormone is produced	Primary or secondary atrophy of pituitary or organ, where hormone is produced

(Continued)

Table 2A Clinical Pathology (*Continued*)

Code	Parameter	Method of determination (examples)	Increased (examples)	Decreased (examples)
Steroid hormones and others	Estrogens	Radioimmunoassay (RIA)	Hypertrophy, hyperplasia, and neoplasia of pituitary Hypertrophy, hyperplasia, and neoplasia of organ, where hormone is produced	Primary or secondary atrophy of pituitary or organ, where hormone is produced
	Progesterone	Enzyme-linked immunoassay		
	Testosterone	(ELISA)		
	Tri-iodothyronine (T3)			
	Thyroxin			

^aSome laboratories use different units, e.g., for concentrations (e.g., RBC: $10^6/\text{mm}^3$).

^bReference values depend, in particular, on species, strain, and age of animals (e.g., 313).

^cReference values depend, in particular, on species, strain, and age of animals. See also [Refs. 8, 10,11, 310, 314–316](#) and, in particular [Ref. 313](#).

^dSpecial investigations, as needed.

Table 2B Clinical Pathology (*Continued*)

Code	Parameter	Analyser/Reagent/Kit (examples)	Interpretation
<i>Urinalysis^a</i>			
VOL	Volume	Measuring cylinder	Low toxicological significance
SP GR	Specific gravity	Clinical refractometer	The higher the volume, the lower the specific gravity
pH	pH	Dip sticks	Low toxicological significance
PROT	Protein	Dip sticks	Present only in case of kidney disease (glomerular leakage)
GLUC	Glucose	Dip sticks	Present only in case of hyperglycemias
KET	Ketones	Dip sticks	Generally only present in fasting condition
UROBIL	Urobilinogen	Dip sticks	Increased in case of hepatic diseases associated with decreased bile secretion
BILI	Bilirubin	Dip sticks	Increased in case of hepatic diseases associated with decreased bile secretion
BL	Blood	Dip sticks	Present, for example, in case of kidney disease (e.g., glomerulopathy)
RBC	Red blood cells	Microscopy	Present in case of glomerular disease or bleeding into urinary system
WBC	White blood cells	Microscopy	Present, for example, in case of infections of the urinary tract
EPITH	Epithelial cells	Microscopy	Increased number of epithelial cells, for example, in case of urinary tract infection
SPERM	Sperm cells	Microscopy	Without toxicological significance

(Continued)

Table 2B Clinical Pathology (*Continued*)

Code	Parameter	Analyser/Reagent/Kit (examples)	Interpretation
CASTS	Casts	Microscopy	Protein casts (amorphous) in case of proteinuria Cellular casts (granular) in case of increased cellular components in the urine (see above)
PHOS	Phosphates	Microscopy	Increased occurrence under various conditions
URATE	Urates		
UR AC	Uric acid		
Ca OX	Calcium oxalate		
AM DEB	Amorphous debris		
BACT	Bacteria		

^aFor reference values see, in particular [Ref. 313](#).

analysis has greatly simplified morphometric measurements by allowing some automation (16). Nevertheless, morphometrical measurements remain time-consuming and cannot be regarded as a routine method. Morphometrical measurements are done on cells in their tissue context and allow estimation of various parameters concomitantly. In particular, digital image analysis also allows assessment of texture characteristics (e.g., nuclei), which are related, among others, to nuclear activity. One of the most important applications of morphometry is determination of the no-effect level, for example, with regard to hypertrophy and/or hyperplasia in critical cases.

- *Flow cytometry* to measure ploidy (number of chromosome sets), receptor density, and to classify immune cells (17–19). Flow cytometric measurements are performed on a large number of cells and various parameters are assessed simultaneously and in short time. The cells can be sorted (e.g., by receptor type) and used for further analysis. The disadvantage of flow cytometry is that cells have to be isolated under disruption of the tissue architecture and that preparation techniques are not trivial.
- Microdissection techniques (20,21) to obtain microscopically well-defined samples of complex tissues (e.g., of single cells) for special investigations including *molecular analyses* (22).

Molecular techniques are important tools for modern pathologists to investigate special questions mainly related to the presence of certain genes (e.g., viral genes) or the expression of genes (gene activation, gene transcription, and protein synthesis) (23–25). Molecular techniques include among others:

- *Polymerase chain reaction (PCR)*: for rapid synthesis of large quantities of DNA fragments using primers and DNA or RNA isolated from tissues and fluid of interest. It involves a repeated process of separating the two complementary DNA strands by heat and synthesizing new complementary DNA strands from each single strand. For example, the synthesized copies are used for in situ hybridization; that is for rendering visible the occurrence and localization of DNA or RNA which contains that particular sequence.
- *Blotting techniques*: Gel electrophoresis for separation and detection of RNA fragments (Northern blot), DNA fragments (Southern blot) and proteins (Western blot) isolated from diseased tissues and compared to standard probes with regard to their electrophoretic characteristics.
- *Toxicogenomics and proteomics* to investigate aspects of gene expression (22,26–31).

2. TECHNICAL POST-MORTEM PROCEDURES

2.1. Introduction

This section provides a general description of routine techniques. The procedures need to be adapted depending on the study type. The main determinants for tissue sampling, processing, and histopathological investigations follow:

Study duration: For *acute* studies histopathological investigations are limited to unclear macroscopic findings, because histopathological findings are generally unspecific (e.g., necrosis and edema) and reflect acute intoxication with failure of various organs. For *subacute/subchronic* (1–3 months) and *chronic* (6–9 months) toxicity studies, extensive histopathological investigations are carried out according to regulatory requirements, as described in [Chapter 10](#). This also applies to *life-time bioassays*, which include additional organs generally not sampled in studies of shorter duration (e.g., lachrymal gland) and additional samples (e.g., additional liver sections).

Route of application: The application site has to be sampled and evaluated carefully. Examples include skin (dermal application), muscle (intramuscular application), subcutaneous tissue (subcutaneous application), vein (intravenous application) (32), nasal cavity (inhalation studies by nasal application) or implantation site (implantation e.g., of chips etc.) (33).

Type of tested compound, device, etc.: In addition to conventional chemical entities, newer types of test material include skin equivalents (34), humanized monoclonal antibodies (mAb) (35), synthetic oligonucleotides (36), gene therapy (37), and other biotechnology products (38,39).

Species: Organ/tissue sampling procedures are significantly influenced by the size of the animal and its organs. While cross sections of the whole left and right liver lobe are taken for rodents, samples, which are much smaller than the whole organ, are preserved from different liver lobes of dogs.

Purpose of investigation: While for a standard toxicity study the procedures are regulated mostly according to ICH guidelines (Chapter 10), the investigator running a mechanistic study for elucidation of unclear findings in a previous study is free to concentrate on the most important parameters and organs/tissues.

The awareness that immunotoxicity can be an issue has significantly increased over the more recent years and requires careful pathological investigation of the hemo–lympho–reticular system, at times complemented by functional tests (40–42).

2.2. Necropsy

All animals in a toxicity study or life-time bioassay are subjected to a postmortem necropsy. After euthanasia, the animal is generally exsanguinated. Consistent

bleeding is important for achieving reproducible organ weights. The animal is then placed on its back and the abdominal and thoracic cavities are widely opened. Experienced technicians generally carry out dissection, but a pathologist must be available for the examination of unclear findings. Macroscopic inspection of the corpse, the organs, and contents (if any) in cavities is very important, as lesions once missed macroscopically are generally not preserved and therefore unavailable for histological examination.

A selection of important organs is weighed (for details see Table 3) using animals from toxicity studies but usually not from animals of life-time bioassays. For reliable organ weights, organs have to be cleaned carefully from fat and adhering water and blood (43). Organ weights are easy to record and can be very helpful; for example, liver weights may be more sensitive than qualitative histopathological evaluation; liver cell hypertrophy of 50% can be easily missed under the microscope, as a volume increase of 20% corresponds to only $\sqrt[3]{1.2}$ in linear dimension, which cannot be recognized without morphometric measurements.

Table 3 also lists *standard organ/tissues* to be sampled according to study type and duration. In addition to this standard list, all macroscopic findings must be sampled.

Sampling procedures influence the result of the histopathological procedure significantly. For example, the number and size of pancreatic islets regulating blood sugar vary according to location within the pancreas. The prostate has various distinct regions, which react differently to toxic injuries and to age. Particularly in rodent species, lesions tend to be small and are therefore often detected only histologically. It is evident that the size of the sample influences the probability of such a lesion being detected. These few examples illustrate that it is of utmost importance that consistent sampling procedures and techniques are used throughout the study and across all dose groups and sexes. Standard sampling procedures are available in the literature (e.g., 44–46). Special procedures are at times necessary for special investigations, such as investigations of nerves (e.g., teased fiber technique) (47).

In addition to organ/tissue samples, smears (e.g., of bone marrow or of a turbid exudate in body cavities) can be crucial for the correct diagnosis of a condition. Smears need to be taken at necropsy on unfixed fresh material.

2.3. Histotechnology

Organs/tissues as specified in Table 3 should be examined histologically as follows:

Rodents:

- Toxicity studies: at least top dose and control group. Examination of organs/tissues from lower dose groups may be limited to gross lesions and to organs/tissues showing possibly treatment-related lesions in the top dose

Table 3 Organ List

Organ	Remarks	Organ weights
Adrenal glands		+
Aorta		
Blood smears	Not required in life-time bioassays, but may be very useful to diagnosing proliferative hematological disorders	
Bone with bone marrow and joint with cartilage	Generally femur. In toxicity studies, often also complete joint preserved and investigated	
Brain	At least three levels to include medulla/pons, cerebellum, and cerebrum	+
Epididymides		(+) sometimes with testes
Esophagus		
Eyes with optic nerves	In rodents, the adjacent Harderian gland should be preserved, at least in life-time bioassays for histological investigation if needed	
Gallbladder	In mice (with liver), dogs, and monkeys	
Heart		+
Kidneys including ureter		+
Lachrymal gland		
Large intestine (cecum, colon)	Rectum should be preserved for life-time bioassays for histological investigation if needed	
Larynx	Including Peyer's patches if relevant	
Liver	At least in life-time bioassays	+
Lung with bronchi and bronchioli		+
		In special studies such as inhalation studies
Lymph nodes (representative LN at least at two locations)	For example, Mesenteric LN in case of infeed/gavage studies and bronchial LN in case of inhalation studies	(+)
Mammary glands	Both sexes. In males: subcutaneous tissue at the site of mammary glands to be preserved for histological investigation if needed	

(Continued)

Table 3 Organ List (*Continued*)

Organ	Remarks	Organ weights
Nasal cavity, nasopharynx, and paranasal sinuses	In inhalation studies. For other application routes at least in life- time bioassays to be preserved for histological investigation if needed	
Nerve, peripheral	For example, sciatic	
Ovaries		+
Pancreas		(+)
Parathyroid glands	In rodents preserved and examined together with thyroid	
Pituitary gland		+
Preputial/clitoral glands	Life-time bioassays only	
Prostate		(+)
Salivary glands (mandibular, parotid, sublingual)		(+)
Seminal vesicles with coagulating glands	Rodents only	(+)
Spinal cord	Generally two to three levels	
Skeletal muscle	For example, biceps femoris	
Skin/subcutaneous tissue		
Small intestine (duodenum, ileum, jejunum)		
Spleen		+
Stomach (forestomach of rodents and glandular stomach)		
Testes		+
Thymus		+
Thyroid gland		+ with parathyroid glands
Tongue		
Trachea		
Urinary bladder		
Uterus with uterine cervix and oviducts		+
Vagina		
Zymbal's gland with external ear	Should be preserved, at least in life-time bioassays for histological investigation if needed	

(Continued)

Table 3 Organ List (*Continued*)

Organ	Remarks	Organ weights
Gross lesions including masses, target organs, application sites		

The organs listed above generally need to be preserved during necropsy and investigated histologically for all types of studies (with exception of acute studies) and all species, unless otherwise indicated.

Note: Organ weights are generally not recorded for life-time bioassays; *Note:* + needed; (+), optional.

Source: For details, see also [Refs. 45, 317–322](#).

- Life-time bioassays: usually all animals (terminal sacrifice, intermediate deaths, and termination in extremis)
- Nonrodents: from all animals and all dose groups including control groups

The following section provides a brief overview of histotechnical procedures, as far as they are important for a basic understanding of the preparation of organs and tissues for microscopic evaluation.

Histotechnology comprises the following steps (48,49):

- Fixation of organs/tissues to prevent autolysis
- Trimming of the hardened fixed samples to appropriate size (difficult with unfixed “soft” organs/tissues)
- Dehydration of the sample, that is replacement of the water in the sample with an organic solvent
- Embedding for light microscopic investigations, most commonly in paraffin wax to increase the consistency of the organ/tissue and thus facilitating the histological sectioning
- Histological sectioning
- Removing of the embedding material to allow coloration of the sections
- Coloration of the section to increase contrast between different cellular components
- Mounting the section on glass slides (for light microscopy) and cover slipping the section for protection and to avoid uneven surfaces distorting the optical image

Each of these steps is explained below in more detail.

To prevent autolysis, proteins of organs and tissues have to be denatured (cross-linked) by so-called *fixatives*. The most common fixative is 40% aqueous paraformaldehyde called formalin, often in a buffered neutral solution. Compared to alternatives such as Bouin’s solution,

formalin offers the advantage that the tissue can be left in the formalin solution without becoming too hard. Generally organs and tissues are immersed in formalin, which then penetrates into the organ/tissue at the rate of a few millimeters over 24 hr. Therefore, samples must be reasonably thin (less than one centimeter) to allow a rapid and therefore good fixation. Other fixatives, (e.g., glutaraldehyde), penetrate tissues at a much slower rate, which allows fixing only thin organ slices. The big advantage of the latter fixative is the much better preservation of the cellular micro-architecture, which makes it the preferred fixative for electron microscopy. In contrast, because of unequal shrinkage of cellular components, formalin creates some artifacts (clefts) in tissues. Such artifacts are generally acceptable in most organs. However, particularly for the following organs and for special investigations, alternative fixatives are used such as various formulas of Zenker's or Davidson's fluid (50) for eyes and bone marrow, Bouin's or modified Davidson's fluid (50,51) fixative for testes or a mixture of formalin and glutaraldehyde for nervous tissue (52). Lungs are often fixed by intratracheal instillation of formalin (53).

Vascular perfusion of the whole animal or parts of it with fixative yields much better results than immersion, as denaturation of proteins occurs almost instantly. Fixation by vascular perfusion is particularly meaningful for special morphological investigations of delicate organs such as the testis (using, for example, Bouin's fixative) or for electron-microscopic investigations using glutaraldehyde (54). However, the perfusion technique is time consuming and is not a method of choice for routine toxicity studies.

Once an organ/tissue is fixed, it can be *trimmed* into a smaller piece that contains the region of interest (generally at the most $15 \times 20 \times 5$ mm). Bones have to be decalcified to allow their sectioning. For the reasons mentioned under sampling procedures, it is important that trimming is done consistently. Standard sampling procedures are available in the literature (44–46,55). The water naturally present in organ/tissue samples has to be replaced generally by a liquid, in which the embedding material is soluble (usually an organic solvent). This process is called *dehydration*.

The trimmed samples are then *embedded* with paraffin wax or a resin. Paraffin is the most commonly used embedding material, it is cheap, easy to handle and for routine purposes absolutely adequate. To embed a tissue/organ, it is immersed in liquid paraffin of approximately 58°C. This temperature results in some distortion of the tissue architecture, which can be avoided by using resins, which harden at room temperature. The latter also tend to be harder than paraffin, which allows cutting thinner sections. This is particularly important for cutting ultra-thin sections for electron-microscopic investigations. However, the stainability of resin sections is much lower than that of paraffin sections, mainly because of the difficulty to remove the embedding resin.

If organs/tissues are frozen and then sectioned, no fixation and embedding are needed. However, the quality of frozen section is generally lower than that of fixed and paraffin embedded material.

Embedded organs/tissues are *sectioned* at a thickness of around 3–5 μ using a microtome and the sections are *mounted* on a glass slide. After removal of the embedding material by solvents, the sections are *stained* to increase contrast and to differentiate between different cellular components. Hematoxylin–eosin (H&E) is the most common stain used for routine paraffin sections. A selection of more specialized stains is listed in Table 4. Enzyme/immunohistochemical stains serve for the investigation of cell kinetics (BrdU, Ki 67, etc.) (56,57). In situ hybridization, a tool of molecular pathology, has gained good acceptance in toxicologic pathology to investigate gene expression.

A summary of basic methods for histopathological preparations is presented in Table 5.

2.4. Evaluation of Histological Slides

2.4.1. Microscopes

The pathologist usually examines histological sections using a transmission *light microscope*. More specialized procedures involve Nomarski illumination, which increases the contrast particularly of membranes and creates the illusion of three-dimensional images (3D). Confocal microscopy is used for optical sectioning of thicker sections ($30 + \mu$) and thus lends itself to the three-dimensional reconstruction of cells and organelles.

Investigation of *fluorescence* is another specialized technique. Fluorescence means that molecules, which are excited by light of one particular wavelength, emit light of a different wavelength. Fluorescence can occur with substances “naturally” present in cells such as some pigments, or can be introduced with fluorescent labeling of cellular structures and molecules (58). For dark field investigations, only light that is deviated by structures such as crystals enters the microscope.

Electron microscopy (EM) uses an electron beam and magnetic lenses, instead of light and optical lenses. Because of the much smaller wavelength of an electron beam, EM allows much higher magnifications than light microscopy. To take advantage of this, sections need to be extremely thin (in the order of Å) to decrease superposition and loss of resolution. Electron microscopes are classified as transmission EM (similar to transmission light microscopy) and scanning EM, mainly used to investigate surfaces by aiming an electron beam on to the surface of the sample from the observer’s side, who then looks at the reflection of this beam (59).

Depending on the organ under investigation and the purpose of the investigation, additional procedures are available, such as 3-D visualization of brain lesions by nuclear magnetic resonance (NMR) (60).

Table 4 Basic Histological Stains (Examples)

Stain	Result	Advantage/use	Remarks
Traditional stains:			
Hematoxylin–eosin (H&E)	Nuclei: blue Cytoplasm: red	Rapid, easy, cheap Works well on formalin-fixed tissue Universal stain	Routine stain in toxicologic pathology Allows, for example, to suspect SER proliferation (cytoplasm turns intensively eosinophilic) or foci of cellular alteration (cytoplasm turns blue because of proliferation of rough endoplasmic reticulum)
Periodic acid Schiff's (PAS) reagent	In combination with hematoxylin: Glycogen: rose/purple Mucin: blue Basement membrane: pink	Particularly for certain storage products and for rendering visible basement membranes	Can be combined with other stains such as alcian blue and methenamine silver, etc.
Klüver Barrera	Myelin: greenish-blue Cells: pink/violet	For nerve fibers	
Sudan red	Neural lipids: red	For demonstration of fat storage	Other Sudan dye varieties: Sudan black, etc. Lipid demonstration requires frozen sections
Cresyl violet	Nissl substance: blue	For staining of nerve cell body	

(Continued)

Table 4 Basic Histological Stains (Examples) (*Continued*)

Stain	Result	Advantage/use	Remarks
Histochemistry (HC):			
Acid phosphatase HC	Lysosomes: black		Also works for EM
Dehydrogenase HC	Mitochondria: dark dense precipitate		Also works for EM
Enzyme/immunohistochemistry:			
Bromodeoxyuridine (BrdU) incorporation	Newly synthesized DNA	Demonstration of cell kinetics without use of radioactivity	BrdU incorporated into replicating DNA can be traced, for example, by fluorochrome-labeled antibody
Ki 67	Ki67 nuclear antigen expressing cells (which is all proliferating cells)	Demonstration of cell kinetics without pre-treatment	Ki67 is demonstrated by specific antibodies, which are then visualized, for example, by a biotinylated second antibody
In situ hybridization:	Known DNA sequences produced by PCR are hybridized with homologous sequences present/expressed in tissues	Demonstration of specific DNA or RNA sequences	Appearance depends on the tracing system

Source: For details, see [Refs. 48, 49, 57, 206, 210, 323](#).

Table 5 Basic Methods for Histopathological Preparations

Fixture			Characteristics of sections				
Application	Type	Embedding material	Thickness (μm)	Specimen size	Quality	Staining	Field of application
Immersion	Formalin	Paraffin	5–7	Regular (generally about $1 \times 1 \text{ cm}^2$)	(+)	Normal stains	Routine
	Bouin's	Paraffin	5–7	Regular	+	Normal stains	Can be used as routine for special organs
		GMA	2	Regular	++	Normal stains	Special investigations
Perfusion	Bouin's ^a	GMA	2	Regular	+++	Normal stains	Research or special investigations
	Glutaraldehyde	Epon, araldite	< 1	Generally about 1 mm^2	++++	Toluidine blue	Research or special investigations

^aMixture of formalin and glutaraldehyde;

Grading: From + (Quality generally acceptable) to ++++ (Quality excellent).

Abbreviation: GMA, Glycol methacrylate.

Source: From Ref. 263.

2.4.2. Nomenclature

The task of the pathologist is to recognize organ and tissue alterations and to record these findings in a reproducible way (48,61,62). The pathologist uses a standardized nomenclature, which is not limited to describing single findings (see [general pathology](#) below), but which summarizes a physiological or pathological status of the organ/tissue or system by using diagnostic terms of diseases. For example, an abscess at a subcutaneous injection site may consist of necrotic fat tissue in the center with leukocyte infiltration, a leukocyte wall around this center with—depending on the age of the lesion—lymphocytic components, some edema and possibly the beginning of a connective tissue capsule.

Over the past 30–40 years, pathologists have agreed to a significant extent on standardized nomenclatures particularly for the diagnostically difficult field of neoplasia (tumors, including precursor lesions such as hyperplasia) (63,64). Examples of the standardized nomenclature systems are: WHO/IARC/RITA nomenclature: The rat (65); The mouse (66); STP nomenclature (67); IARC nomenclature for rats (68) and mice (69); monographs on the pathology of laboratory animals (70) as well as further books (71,72).

2.4.3. Control Data

For toxicity studies, the pathologist generally examines the animals on an organ-by-organ basis, while for life-time bioassays the pathologist looks at all organs/tissues per animal. The first approach allows a good comparison between control groups and dose groups and sexes, while the latter provides a better picture of the disease status of any particular animal, which is important for life-time bioassays.

A toxicity study or rodent life-time bioassay is an epidemiological study, where findings in dosed animals are compared to those in the control group(s). Therefore, it is important that incidental or background findings and lesions are recorded (48) with regard to their occurrence, severity and—if multiple time points of investigation are available—their first observation and further development. Many findings are not of pathological significance. For example, lymphocytic foci in or under the intestinal mucosa are normal, but their occurrence and extent might be increased in case of irritant compounds. The *concurrent control* is the best control. Sometimes, the concurrent control group may show an unusual incidence of a particular lesion (low or high) or rare findings not present in the concurrent control group may be found in one or the other dose group. This is particularly important for the evaluation of results from life-time bioassays. Under such circumstances one might want to use *historical control data* from the same strain and facility. Ideally these data should not be older than 5–8 years, because of the genetic drift and evolution of diagnostic nomenclature. If

insufficient data are available from the same facility, one might consider using data from a *historical control database*. Good historical control databases share the following characteristics (64,73–78):

- Quality control (consistent nomenclature, peer review, and data check)
- Data on possibly modifying factors, such as strain, breeder, husbandry, age of the animals, sampling, and histotechnical procedures
- Audit trail for changes of nomenclature over time available
- Data on stability of tumor pattern over time
- Visual documentation of characteristic lesions in the database is a special asset

2.5. Statistical Testing

Incidences of histopathological observations in *toxicity studies* are generally analyzed only by biostatistical methods (e.g., *t*-test) for their statistical significance in case of a problem with interpretation.

For *life-time bioassays*, the following tests are routinely used (76,79–84):

- Fischer test: pair-wise comparison; good for small numbers
- Peto test: survival-adjusted trend test (asymptotic form) stratified according to time (often in 4 periods); need to distinguish fatal and nonfatal tumors; needs larger number of animals/test group (50+) (85)
- Exact Peto: combination of Peto and Fischer test; good for small numbers
- Cochran–Armitage test: trend test (simpler than Peto test); no need to determine cause of death; often used for nontumorous lesions.

It is important to remember that *statistical significance* only flags out a particular finding, but does not tell anything about its *biological significance*. Biological significance can only be derived from sound scientific judgments taking into account all information available about the observation, the experiment as such and scientific knowledge at large.

2.6. Quality

Quality assurance is also highly important in pathology. The general good laboratory practice (GLP) rules have to be observed.

The pathologist can judge the *quality of the test animals* with regard to their health, in particular the occurrence of subclinical infections. As will be explained later, the latter are evident from the incidence and size of inflammatory foci (e.g., in livers and lungs).

Tissue accountability is an important factor to judge the quality and validity of toxicity and in particular of life-time bioassays (48,86). It is influenced by various factors, such as autolysis, age of the animal (e.g., thymus), size of organ, amount of tissue available and preserved during necropsy, and embedded in paraffin blocks. There are no guidelines, but a recommended minimum standard for life-time bioassay studies is 90%/sex/group with the following exceptions or specifications (75):

- Parathyroid glands: for at least 70%/sex/group sufficient tissue from at least one gland
- Adrenal glands—cortical and medullary tissue available bilaterally in at least 70%/sex/group and unilaterally in 90%/sex/group
- Thymus: for at least 80%/sex/group
- Mammary gland—rat
Males: for at least 90%/group a section of subcutaneous tissue from the mammary area (caudal portion of the ventral abdomen) with or without male mammary gland
Females: for at least 90%/group subcutaneous tissue with mammary gland components
- Mammary gland—mouse: similar to rat, but for both males and females at least 70%/group

Blinded slide evaluation means evaluation of slides without knowing the treatment group. It is not recommended for routine examinations (87). However, blinded reading of an organ with an equivocal or subtle finding can be very helpful. If done, it should be recorded in the raw data and the study report and this will enhance the confidence of the regulatory reviewer on the validity of the data (75).

In addition, *peer reviewing* has become a standard procedure in pathology (88–90). Specifically, a pathology colleague examines a selection of the histological slides of a study. This can mean that 10% of all animals at random plus all target organs are re-examined by the reviewing pathologist. The procedure has to be recorded; in particular discrepancies and their resolution should be noted.

3. GENERAL PATHOLOGY

An organism and its organs/tissues have a limited spectrum of reactions to non-physiological conditions, including toxicity and carcinogenicity.

This section defines the basic pattern of reactions, provides some insight into the pathogenesis of these basic lesions, and addresses their consequences as far as relevant for toxicology.

3.1. Type of Lesions

The basic types of pathological lesions are summarized in Table 6 and selected examples from rat studies (exception Fig. 2: monkey study) are shown in Figs. 1–10.

The following lesions are frequently seen in toxicity studies:

- *Regressive alterations* including, cytotoxicity, and cell death, the classical endpoints of acute to subacute toxicity.
- *Progressive alterations*, including repair processes following toxic injury as well as adaptive changes such as hypertrophy (increase in cell size) and hyperplasia (increase in cell number). Progressive changes are important particularly in long-term studies, especially in life-time rodent bioassays. Irrespective of the underlying pathogenesis, cell proliferation observed in shorter term studies generally indicates an increased likelihood for finding tumors in life-time rodent bioassays at comparable doses (91–97). This is related among other factors to an increased risk of proliferating cells to become genetically abnormal (point mutations, chromosomal abnormalities, etc.). For example, various substances bind to $\alpha_2\mu$ -globulin in renal tubules, thereby leading to kidney toxicity with necrosis of single tubular cells and tumors in rats (98,99). However, cell proliferation is not always followed by neoplastic changes (100).
- Signs of *inflammation*, be it as consequence of small infections (particularly in nonrodent species) or following toxic injury with cell death
- Alterations associated with *metabolic changes*, which includes storage of various endogenous substances (e.g., fat) and of administered chemicals
- *Circulatory and respiratory disturbances* are less frequent, but can be observed, as in the case of arteritis and phospholipidosis
- *Malformations* are the endpoint of reproductive toxicity studies (not addressed here), but can also occur spontaneously

3.2. Some Important Subcellular Alterations

Toxic injury may be first seen in the EM at the subcellular level, before histopathological changes are evident under the light microscope, which then later might result in macroscopic lesions (see also below).

The following organelles react most frequently to exposure to chemicals:

- The *smooth endoplasmic reticulum (SER)*, particularly of the liver, is the site of metabolizing enzymes, among them also those metabolizing exogenous chemicals. Exposure to chemicals is often
- (text continued on p. 450)*

Table 6 General Pathology

Lesion	Definition	Examples	Regulation/Pathogenesis	Consequences	Remarks/ Interpretation
1. Malformations and aberrations	Malformation = teratogenic effect: often a consequence of incomplete organogenesis (6)	Cleft palate	Essentially by exposing germ cells, embryos, and—with lower probability—fetuses to chemicals (see textbooks of reproductive toxicity). The molecular basis of malformations is essentially: – Genetic alterations (mutation, chromosome aberration, etc.) – Interference with vital cellular processes by chemicals, radiation, etc.	Malformations can be insignificant (e.g., additional toe) to lethal (e.g., missing closure of the neural crest)	As only healthy animals are selected for toxicity and carcinogenicity studies, significant malformations are not seen
	Aberration: e.g., remnants (324) and ectopic tissue (325,326)	Cyst with respiratory epithelium in thymus, misplaced hepatocytes or pancreatic tissue, etc.	Spontaneous	Ectopic tissue is generally without physiological significance	—
2. Regressive alterations					
2.1. Atrophy	Loss of substance: e.g., cell volume, organ size, etc.	Age-related atrophy of the thymus and other organs in adult animals	Physiological involution and/or decreasing protein synthesis with age (e.g., because of tighter binding of histones to DNA)	Reduced functional spare capacity of organ	Lymphatic and hemopoietic tissues are regularly atrophic in animals from life-time studies

2.2. Apoptosis	Orderly cell death (cell turnover)	Pressure atrophy: e.g., atrophic tissue around growing tumor	Among other effects: pressure leads to diminished vascular perfusion	If extensive, can lead to failure of the organ	Formulation of tumor capsule, which, however, offers little protection against tumor spread
		Hunger atrophy (inanition)	Reduction of vital processes, lack of essential nutrients, etc.	Can lead to death	Can occur in high-dose animals of toxicity studies to various degrees
		Hormonally induced atrophy: e.g., steroid-induced atrophy of the lymphatic tissue	Physiological receptor-mediated effect	For example, increased susceptibility for infections	Partly reversible upon session of exposure to substance
2.3. Necrosis	Cell death	In many organs, e.g., in the intestine	Spontaneous or induced. Starts, for example, with increased intracellular Ca and activation of endonuclease	Not accompanied by inflammation	Apoptosis is a physiological process to remove old or damaged cells, including neoplastically transformed cells
		Any organ	Can be the ultimate consequence of a series of alterations starting with reversible changes (e.g., cellular swelling or fatty accumulation) At the molecular level: disturbance of DNA expression, inhibition of vital enzymes, disturbance of membrane integrity, etc.	Depending on extent and organ: can be followed by complete or partial reparation, in the latter case with fibroblast proliferation and scar formation	Depending on extent and organ, death of parenchymal cells is always a significant finding. Particularly easy to see with acute toxicity. However, single cell necrosis also occurs in long-term toxicity studies

(Continued)

Table 6 General Pathology (*Continued*)

Lesion	Definition	Examples	Regulation/Pathogenesis	Consequences	Remarks/ Interpretation
3. Progressive alterations					
3.1. Parenchymal regeneration	New cells are of the same kind as replaced cells	Spontaneous shedding of intestinal epithelia and replacement by cells from the crypts Cell division in target organ toxicity, e.g., in liver following hepatotoxicity with single cell necrosis	Physiological process to replace, e.g., aging cells Activation of undifferentiated hepatocytes	Maintenance of functional integrity. Tissue does not change Restitutio ad integrum, but increased cell proliferation is often associated with tumor formation in life-time rodent bioassays	All cells with exception of nerve cell bodies can regenerate
3.2. Scar	New cells are different from replaced cells	Wound healing	Activation of fibroblasts to form granulation tissue, which produces connective tissue fibers	Incomplete repair	
3.3. Hypertrophy	Increase of cellular volume because of increased organelle numbers ^a	Hepatocellular hypertrophy	Adaptive change generally to cope with increased workload, e.g., metabolism of exogenous substances	Organ weight increased. Fully reversible, if underlying condition ceases (e.g., following termination of exposure to chemical)	Hypertrophy per se is not a sign of toxicity but a physiological response (233)

3.4. Hyperplasia	Increase in cell number in an organ Cells have normal appearance	Hepatocellular hyperplasia	Mitogenic stimuli following increased workload, cytotoxicity, or genetic toxicity		Generally reversible. Hypertrophy and hyperplasia often occur concomitantly and are difficult to distinguish
3.5. Neoplasia	Autonomous growth of a tissue Cells often structurally abnormal (e.g., larger nuclei with different texture, etc.)	Liver cell adenoma	Autonomous mitogenic stimuli with loss of contact inhibition of growth, therefore compression of surrounding tissue, resulting in capsule formation	May lead to atrophy or necrosis of significant portions of the organ because of compression	Irreversible (though some tumors, which fulfill the histological criteria of a tumor, were found to regress following termination of exposure to chemicals)
		Liver cell carcinoma	In addition, production of proteases, resulting in infiltration into surrounding tissue and vascular vessels leading to distant metastatic foci of tumor cells	Because of infiltrative and rapid growth often fatal	Irreversible
4. Alterations associated with metabolic changes	Often degenerative changes, where cellular processes become defective	Vacuolar cell degeneration, for example, of renal tubular epithelia	Activation of oncogenes Barrier function of the cell membranes disturbed, for example, because of insufficient production of ATP in mitochondria	Initially reversible Can progress to cell death	Edema formation and vacuolar cell degeneration are seen in acute intoxication and conditions such as intestinal ulceration, chronic inflammation, etc.

(Continued)

Table 6 General Pathology (*Continued*)

Lesion	Definition	Examples	Regulation/Pathogenesis	Consequences	Remarks/ Interpretation
		Deposits of endogenous or exogenous material	Endogenous material: deposits of pigments (insoluble proteins increasingly formed with age), fat, immunoglobulins, etc. Exogenous material: deposits; eg., of test compound on its metabolites	In severe cases, cell function can be reduced, for example, reducing metabolic exchange between cells and blood	Age-related pigments (lipofuscin) are regularly seen in older test animals. Occurrence and extent are sometimes increased in toxicity studies without obvious consequences
5. Circulatory disturbances	Insufficient perfusion of an organ/tissue with blood	Arteritis	Narrowing of the vascular lumen leads to insufficient perfusion	Infarction of parts of an affected organ (organ failure or partial restitution with scars)	Circulatory disturbances are not frequent in toxicity studies
		Persistent tachycardia in dogs treated with vasoactive drugs	Vascular perfusion is normal but insufficient to cover the increased demand for oxygen	Scar formation	Is not predictive for man, as this degree of tachycardia is tolerated by man

6. Respiratory disturbances	Disturbance of gas exchange in the lungs between the alveoli and the blood vessels	Spontaneous alteration: pneumonia	Bacterial and/or viral infection	Can heal or be lethal	Respiratory disturbances are not frequent in toxicity studies
		Induced for example, by mistake during gavage of liquid test formulation into the trachea	Irritation of bronchial and alveolar epithelium by chemical(s)	As above	Can happen even if the technician who treats animals by gavage is experienced
7. Inflammation	Characterized by leuko/lymphocytic infiltrates, hyperemia, and edema	Abscess formation in subcutaneous tissue, for example, following subcutaneous infection	Infection (bacteria, virus, fungi)	Can be accompanied by fever. Generally painful and impairing the function of affected organ	Inflammation is frequently seen as a secondary phenomenon in toxicity studies
		Pneumonia (see above)	Chemicals (irritation) As a consequence of cell necrosis	Often restitutio ad integrum	

^aIncreased cell volume because of excessive storage, eg., fat, is not called hypertrophy.

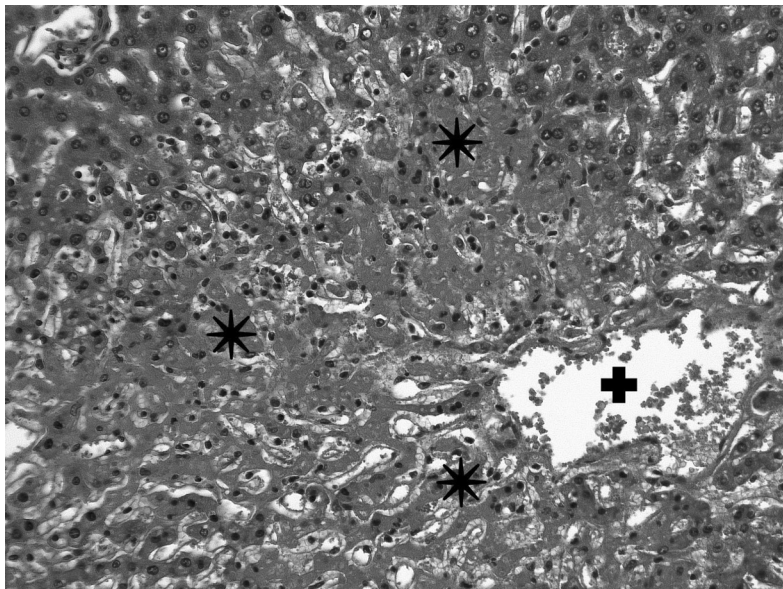


Figure 1 Liver: Focal necrosis—A focus of homogenous eosinophilic (pink) necrotic hepatocytes (*) adjacent to a lobular central vein (+). Note loss of normal hepatocellular structure and round nuclei, while elongated nuclei of sinusoidal cells lining the blood spaces are still present. (See color insert [p. 1.](#))

accompanied by a proliferation of SER. Chemicals of the barbiturate-type can lead to spectacular SER proliferation (14), which microscopically translates as cellular hyperplasia and manifests itself by eosinophilia and at necropsy by increased organ weights. Life-time studies with significant SER proliferating agents generally result in liver tumors in rodents, which are of no significance for man (101).

- Lysosomes take up especially exogenous substances by a process called endocytosis. Lysosomes are particularly abundant in cells specialized in phagocytosis (e.g., leukocytes and macrophages). Lysosomal enzymes in these so-called phagolysosomes can often degrade and thus remove the ingested material. If the endocytosed material is not degradable, the resulting phagosomes can accumulate in the cell or be excreted from the cell to be transported by the lymphatic system and accumulate in lymphatic organs (e.g. spleen or reticuloendothelial system of the liver). Significant accumulation of phagosomes is called a storage-type disease, which can impair the physiological functioning of the cell and even lead to single cell

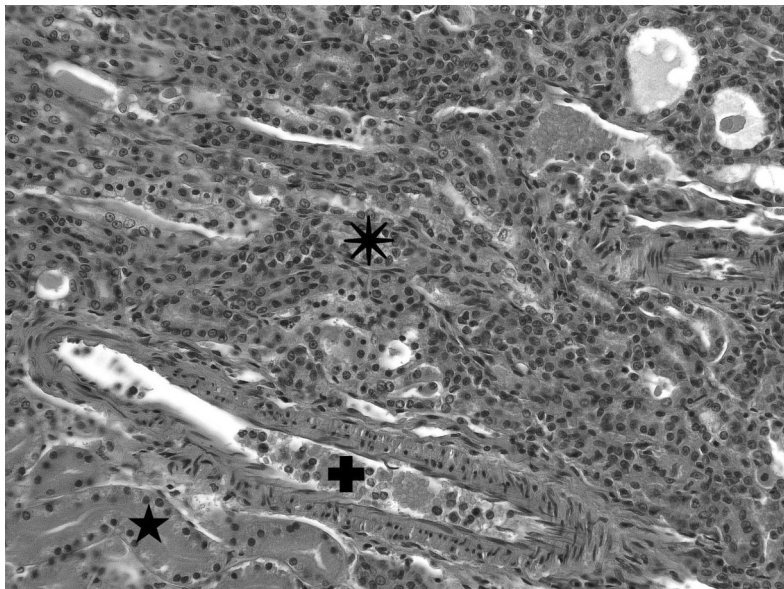


Figure 2 Kidney: Regenerating proximal renal tubules—In contrast to normal eosinophilic tubules (★), regenerating tubules (★) are basophilic (blue) and cellular (higher cell density). On this microphotograph normal and regenerating tubules are separated by a renal artery (+). (See color insert [p. 1.](#))

necrosis. Drug-induced storage disorders can also occur in other organs including kidneys (102).

- *Peroxisomes* are small vesicles in the liver, which contain among others peroxide reducing enzymes. There is a whole class of chemicals, the so-called peroxisome-proliferating chemicals, which makes these peroxisomes proliferate. In life-time rodent bioassays this class of compounds generally induces liver tumors which are considered of little to no significance for man (97,103,104).
- Isolated reports (personal communications and observations) indicate that *other subcellular organelles* can also proliferate under chemical exposure, among them are mitochondria. The toxicological significance is not known.

3.3. Time-course of Lesions

A toxic injury generally *starts* with functional impairment (e.g., cellular membrane impairment), which then leads to structural changes such as microvesiculation (accumulation of water, in mitochondria or lysosomes for example), and later becomes visible under the light microscope as vacuoles (generally empty spaces).

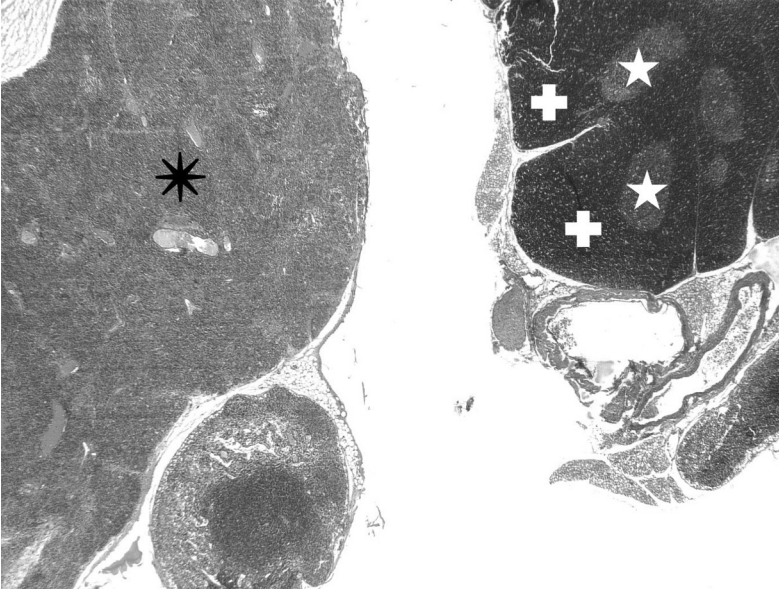


Figure 3 Thymus: Atrophy (*)—Note loss of structure in comparison with normal thymus, where cortex (+) and medulla (★) can be clearly distinguished. (See color insert [p. 2.](#))

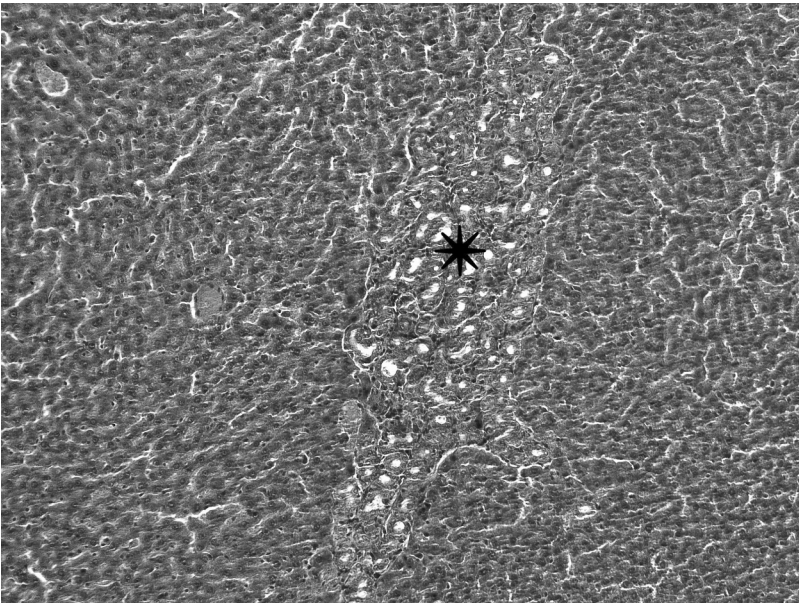


Figure 4 Liver: Bile duct hyperplasia—A focus of proliferating bile ducts (*) surrounded by normal hepatic parenchyma. (See color insert [p. 2.](#))

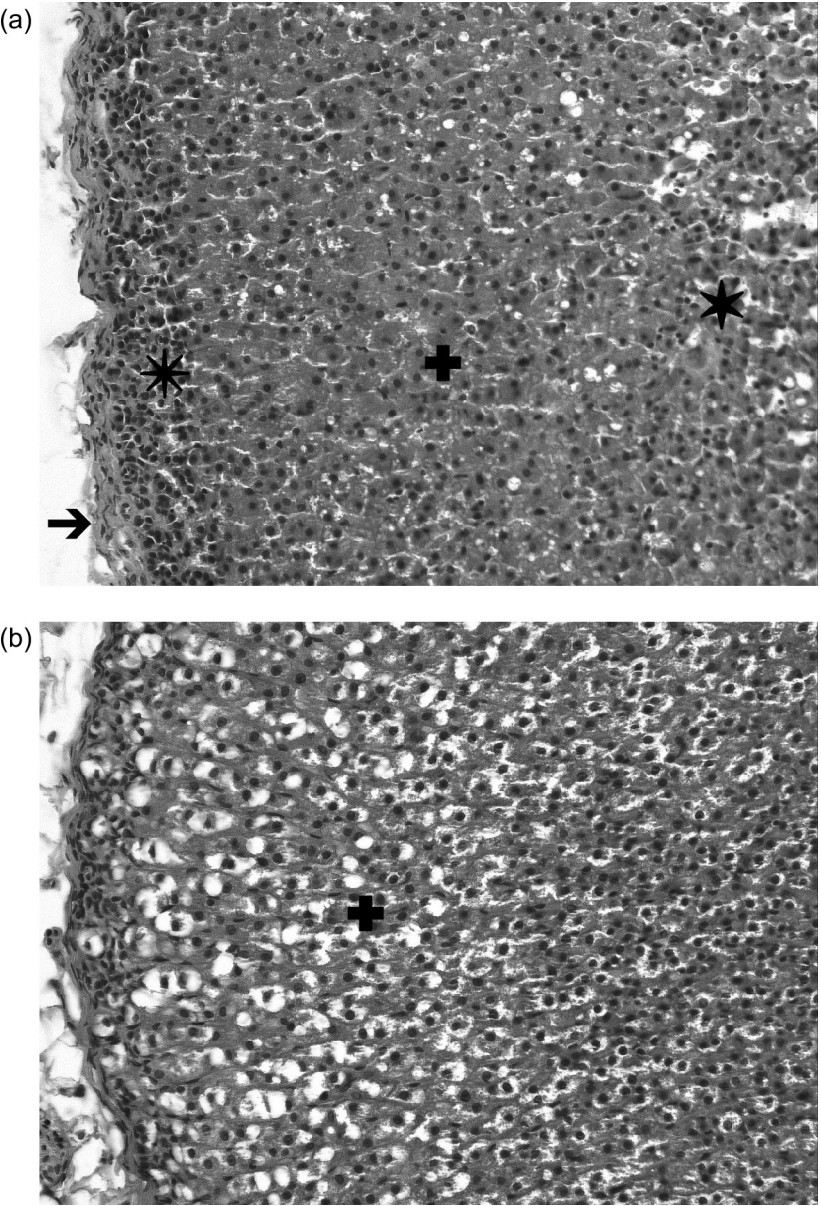


Figure 5 (a) Adrenal cortex: Normal—Note the appearance of the three cortical zones, namely the zona glomerulosa (*), zona fasciculata (+), and zona reticularis (*). → = adrenal capsule, and (b) Adrenal cortex: Cortical hypertrophy—Cells of the zona fasciculata (+) are enlarged and vacuolated (optically empty spaces, as the lipid was removed during histological processing). (See color insert [p. 3.](#))

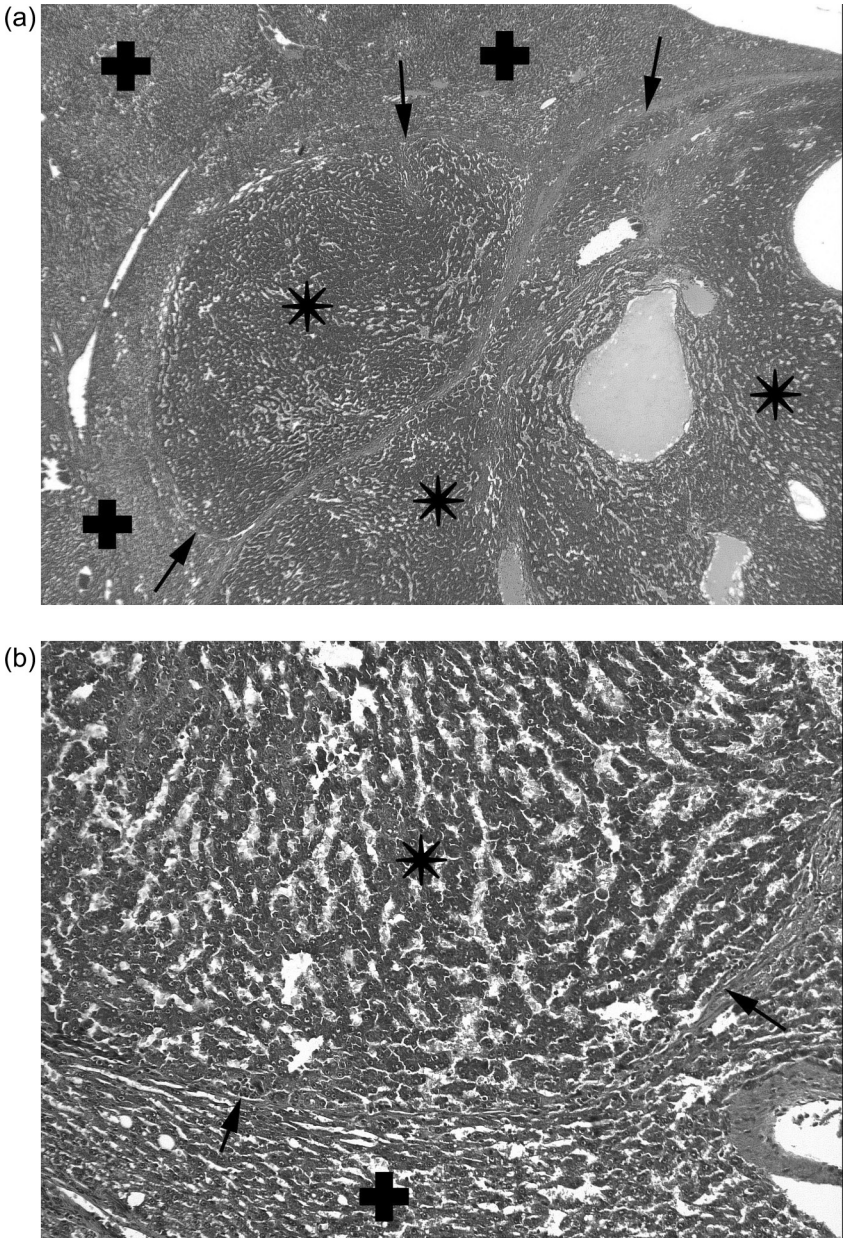


Figure 6 (a) Liver: Adenoma (low magnification)—Large areas of proliferating cells (*) with some sinusoidal structure (vascular spaces), compression of normal hepatic parenchyma (+) and partial capsule formation (→), and (b) Liver: Adenoma (higher magnification)—Note the clear delineation between adenoma (*) and normal compressed parenchyma (+). Fibrous strands of early capsule formation (→). (See color insert [p. 4.](#))

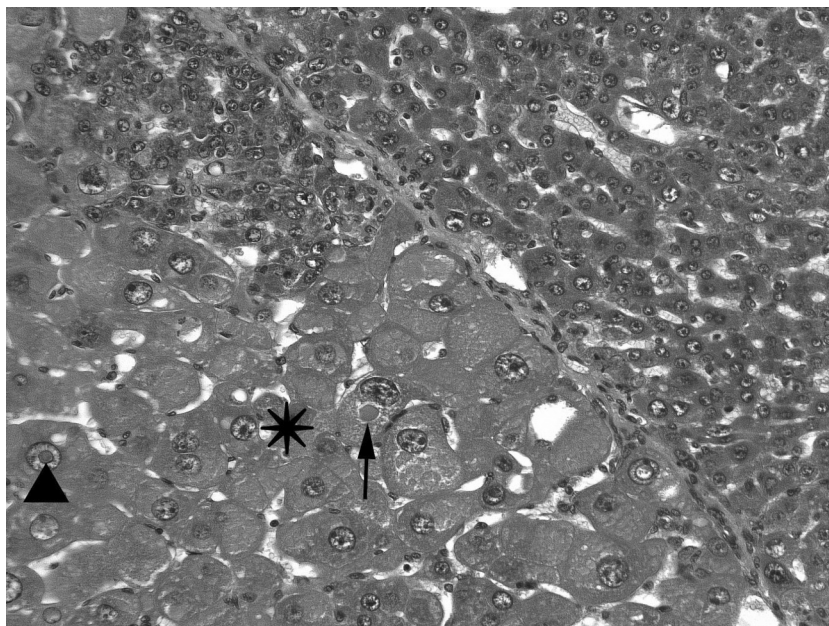


Figure 7 Liver: Carcinoma—High magnification of a hepatic carcinoma (*) showing mostly large eosinophilic (pink) tumor cells with nuclei of variable size (nuclear pleomorphism), intranuclear (▶) and intracytoplasmic (→) inclusions and lack of clear sinusoidal structure. Mitotic figures are not apparent on this photomicrograph. (See color insert p. 5.)

If the injury *persists*, cell organelles might be disrupted and the cell might eventually die. Cell necrosis liberates chemical substances, which attract leukocytes and possibly macrophages, which will then phagocytose the cell debris. Lymphocytes generally appear somewhat later in the process, but often stay longer at the scene and are therefore indicative of a process, which is a few days (or more) older.

However, early toxic lesions may disappear under continuation of exposure to a particular chemical because of *adaptation* of the organism. Thus, partial acute tubular necrosis in kidneys of various species can be seen initially, but already after one or two weeks of exposure the kidney appears again normal, with the exception of tubular basophilia in H&E sections, which characterizes the regenerated tubular cells.

With *progressing severity* of a lesion, the likelihood of complete repair decreases. Thus, early lesions are generally fully reversible, if exposure to the toxin ceases. Cell death is per se an irreversible process. However, some tissues, including the liver and kidneys, have a high capacity for regeneration. Repair can be complete (*restitutio ad integrum*) or partial. In the latter

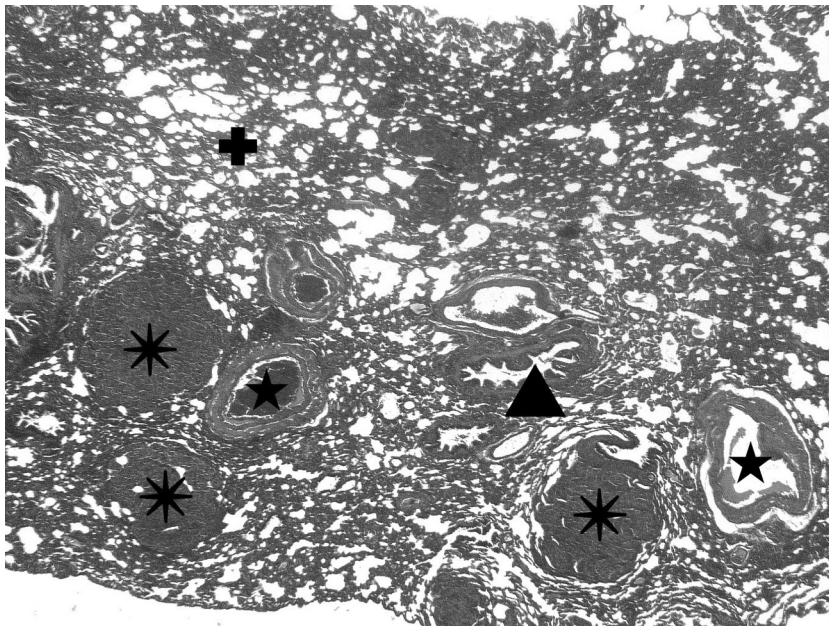


Figure 8 Lung: Metastases of fibrosarcoma (*)—Note also normal lung parenchyma (+), large blood vessels (*), and airways (▶). (See color insert *p. 5*.)

case, replacement with regular cells is achieved only partially and the remaining defect is filled with unspecific cells of the connective tissue type called fibroblasts, which produce connective fibers and mature to fibrocytes.

In selected cases, tumor development in internal organs can also be observed in living animals using suitable techniques (e.g., NMR) (105).

3.4. Modifying Factors

There are a number of factors, which can modify the outcome of toxicity and in particular of a life-time rodent bioassay studies (106–110). The most important factor for the latter is *feeding* (106,108) and standardization of procedures (111). Feeding *ad libitum* significantly increases tumor incidences of the pituitary, the mammary gland, and the lung in rats and of the liver in mice (112–116). However, the incidence of uterine tumors in rats may decrease (116). Feeding *ad libitum* also significantly increases the incidence and severity of degenerative diseases, including nephropathy, myocarditis, polyarteritis, and prostatitis (117). Nephropathy might significantly impact the excretion of the test compound. Overall, feeding *ad libitum* shortens the life span of test animals.

By contrast, reduced feeding (118,119) and feeding *ad libitum* of modified diets rich in fibers (120) significantly decreases incidences of most

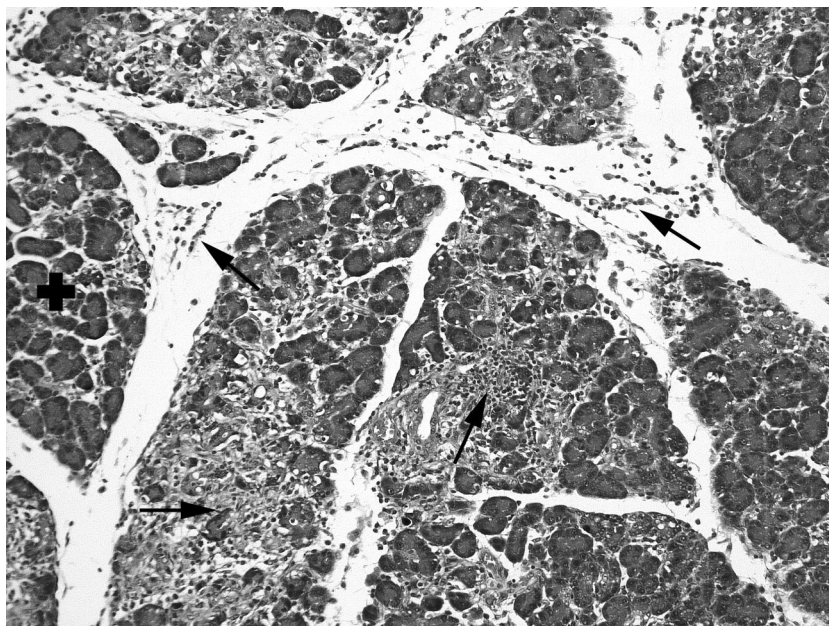


Figure 9 Pancreas: Inflammation—Inflammatory cells (→) infiltrating into and around pancreatic lobules. Note also some exocrine atrophy in comparison with normal portion of a lobule (+). (See color insert [p. 6.](#))

proliferative lesions, in particular those of endocrine organs. It also lowers the occurrence and severity of age-related degenerative diseases, but may increase the incidence of tumors of testis and uterus in rats (116). Caloric restriction can decrease the metabolism of xenobiotics and herewith modify the outcome of life-time bioassays (121).

Toxicity is generally associated with decreased *well-being* of the test animals and therefore with decreased feed intake. In addition, poor palatability of the test compound given by feed admixture reduces feed intake. Not infrequently and despite careful dose-selection to produce only minimal signs of toxicity, this may happen in life-time bioassays (122), thus biasing their outcome for the reasons explained above. Signs of reduced feeding and of unspecific toxicity are also seen in shorter-term studies and consist of the following:

- Retarded growth of young animals
- Weight loss (in certain cases)
- Decreased organ weights, particularly of the lymphatic system (thymus, etc.)
- Decreased resistance to infections with multiple foci of acute to chronic infection. Such infections are not such infrequent,

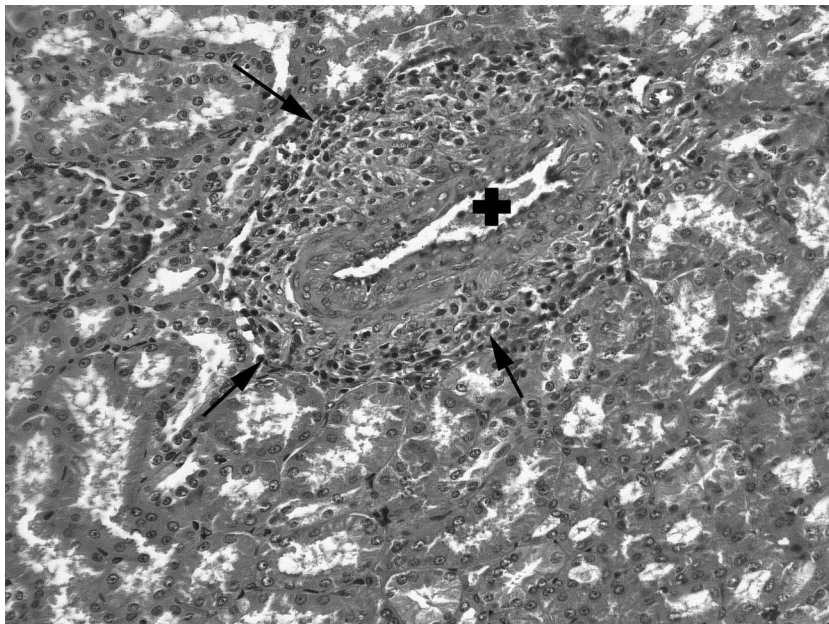


Figure 10 Kidney: Vasculitis—Inflammatory cells (→) mainly around a small blood vessel (+). The surrounding renal tubules are normal. (See color insert *p. 6.*)

particularly but not exclusively in nonrodents, and can jeopardize the outcome of toxicity studies (123–125).

4. TYPICAL NON-NEOPLASTIC ALTERATIONS SEEN IN TOXICITY STUDIES

4.1. Organ Toxicology

Typical *nonneoplastic alterations* seen in toxicity studies with rodents, dogs, monkeys, and minipigs are summarized in [Table 7](#). For further details, a number of good histopathology atlases (70–72,126) and textbooks (127,128) are available.

Toxicity manifests itself often in single *target organs*, but can also affect whole systems, (e.g., the reticuloendothelial system). For this reason, storage diseases (e.g., phospholipidosis) are seen throughout the body and are manifest in lung, liver, lymphatic system, and partly also in other organs (129).

The target organ also depends on the physical characteristics of the test compound. This is best illustrated by inhalation studies: test compounds in powder form with particle sizes $>5\mu$ are deposited in the nose and, therefore, may exert an irritative potential only locally. Particles of $1\text{--}5\mu$ deposit in the airways and $<1\mu$ particles reach the alveoli (130,131).

(text continued on *p. 491*)

Table 7 Typical Non-neoplastic Toxicological Lesions^a

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
1. Liver					
<i>1.1. Hypatocyte</i>					
1.1.1. Storage	Fatty change	Can have many reasons, such as organelle injury, metabolic disorders, etc.	Organ weight increased Per se reversible If excessive: liver cell function disturbed	Ethionine overdosage	Frequent, unspecific sign of toxicity
	Glycogen storage	Higher than normal storage of glycogen	Organ weight increased Per se reversible. If excessive: liver cell function disturbed	Acetaminophen	Unspecific, frequent finding in toxicity studies
	Other cytoplasmic inclusions	For example, multilamellar bodies resulting from undegraded membranes	If excessive: liver cell function disturbed	For example, tricyclic antidepressants (331)	
	Hepatocellular and sinusoidal pigmentation	Storage of Fe, lipofuscin (an age-related natural pigment), hemosiderin (e.g., after hemolytic anemia), test compound	Probably none	Many different compounds, e.g., vincamine alkaloids	Normal with increasing age, but can be increased after exposure to chemicals

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
1.1.2. Nucleus	Increase in size and/or number	Hyperploidy of hepatocyte nuclei or fusion of hepatocytes to form multinucleated cells	—	Nitro compounds, pyrolizidine alkaloids	Unspecific reaction of hepatocytes to hepatocellular injury
1.1.3. Regressive changes	Hydropic/cystic degeneration	Intracytoplasmic accumulation of fluid Partly probably derived from Ito cells (332)	Can lead to cell death	Radio-opaque agents	Relatively frequent, unspecific sign of toxicity
	Single cell necrosis	Cytotoxicity	Liver enzymes elevated Regeneration by proliferation of hepatocytes can lead to hepatocellular tumors in life-time bioassays	Many chemicals after high dose, e.g., acetaminophene	Can be monitored in man by checking liver enzymes (see also the section “Clinical Blood Chemistry” in Table 2) To be distinguished from apoptosis
1.1.4. Progressive changes	Hypertrophy	For example: – proliferation of: smooth endoplasmic	Increased liver weight Eosinophilic hepatocellular cytoplasm	Barbiturates (SER) Some lipid lowering drugs (peroxifomes)	Relatively frequent, unspecific adaptation to chemical overload,

		reticulum (SER) (14,101,235) – peroxisomes (97,103,104,264, 265,274)	Per se reversible. In rodent long-term studies generally associated with liver tumors		often with hepatocellular hyperplasia, particularly centrilobular
	Foci of cellular alteration (333–339)	Various types; the basophilic type is characterized by proliferation of the rough endoplasmic reticulum	Highly active cells with increased protein synthesis and generally increased mitotic index May lead to liver tumors in long-term studies	Many animal carcinogenes, which are not mutagenic	The basophilic type is considered preneoplastic, but is essentially still reversible Can also occur spontaneously at lower incidence in older rodents
	Nodular hyperplasia	Nodular hyperplasia of hepatocytes, for example, following longer-term liver toxicity	May result in impaired liver function and hepatic blood flow	Many liver toxins	Generally accompanied by fibrosis
1.2. Bile ducts	Cholestasis	Bile retained in hepatocytes, Kupffer cells, and bile canaliculi, most likely because of increased bile viscosity and/or impaired bil excretion	Increased alkaline phosphatase “Jaundice” Depending on severity: any outcome possible from recovery to liver failure	Experimental antidepressive agents, griseofulvin (mice)	Rare in animals, but relatively frequent as side-effect to drugs in man

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
	Cholangitis	Inflammation of the intra- and sometimes the extrahepatic bile ducts. Acute or chronic	May lead to cholangiofibrosis, associated with significant impairment of liver function	Phytotoxins and mycotoxins	Relatively rare finding in toxicity studies
	Hyperplasia	Proliferation of bile duct epithelia sometimes accompanied by periductular inflammation and fibrosis	Outcome mainly determined by underlying disease	Many compounds	Frequent, particularly in rats, sometimes also in dogs
1.3. Sinuses	Sinus dilatation	Cardiomyopathy with resulting congestion of the liver	Severe liver congestion results in impairment of the liver	Daunomycin	Also a consequence of hepatocellular atrophy because of unspecific toxicity or as terminal hemostasis
1.4. General	Foci of chronic inflammation	Spontaneous infections	None, if present in small number		Incidence is a measure for the quality of the test animals

	Cystic degeneration	Possibly arising from altered fat storing perisinusoidal cells	Loss of liver parenchyma	Nitroso compounds	Seen particularly in long-term rodent studies
	Fibrosis	Proliferation of fibroblasts with increased formation of connective fibers, often creating a nodular appearance of livers	May progress to liver cirrhosis with significant impairment of liver function	Many liver toxins	Incomplete regeneration after liver toxicity
2. Alimentary system (340)					
2.1. <i>Mouth</i>	Hyperplasia of gingiva	Not known	Impairs feeding	Cyclosporine Ca-channel blockers (341)	Sometimes not fully reversible
	Alterations of teeth	Cytotoxicity of tooth-forming cells Hypercalcification	Impairs feeding	Antitumor agents (342) PTHrP (343)	
2.2. <i>Salivary glands</i>	Acinar hypertrophy	Stimulation of sympathetic innervation	Can lead to acinar necrosis upon prolonged treatment	Isoproterenol	Reversible if not accompanied by acinar necrosis
	Acinar atrophy	Unknown	Feeding and digestion can be impaired	Some antineoplastic agents	Also seen in animals in bad general condition for prolonged time
2.3. <i>Stomach</i>	Proliferative lesions of the forestomach	Especially hyperplasia of the keratinized stratified squamous	None	Many different chemicals, e.g., antineoplastic agents	Hyperkeratosis also seen in rodents with depressed feeding

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
		epithelium because of irritation following gavage or in-feed administration of test substances			
	Proliferation of enterochromaffin-like cells (ECL)	Therapeutic achlorhydria (no gastric HCL) results in hypergastrinemia, which exerts a constant stimulus on ECL	Can give rise to carcinoids (ECL tumors) and adenocarcinomas in long-term rodent bioassays	H ₂ blockers	Regarded as not being significant for man (275–277)
	Ulcers (missing epithelium with necrosis of underlying tissue and inflammation)	Inhibition of prostaglandin formation with decreased formation of mucus, which protects the epithelium against digestion by gastric and intestinal enzymes	Impairs the general well-being Can lead to gastrointestinal hemorrhage and perforation Accompanying inflammation can result in intra-abdominal adhesions	Non-steroidal anti-inflammatory drugs (344) Steroids (253)	Ulcers are sometimes very small and only detected microscopically Ulcers can also occur in the small intestine

		Other mechanisms (e.g., mucosal hemorrhage) also occur			
2.4. Intestine	Villus stunting, particularly in small intestine	Cytotoxicity of mucosal and, particularly, of crypt cells	If minor: reversible If extensive: lethal	Cytostatics	Proliferating cryptal cells are particularly sensitive
2.5. <i>Pancreas</i>					
2.5.1. Exocrine					Toxicologically relevant non- neoplastic changes are rare in the exocrine pancreas See endocrine system
2.5.2. <i>Endocrine</i>					
3. Urinary system (117,247,248,345–353)					
3.1. <i>Kidney</i>					
3.1.1. Glomerulus ^b	Cytoplasmic vacuolation	Early sign of tubular toxicity: increased membrane permeability lead to “osmotic nephrosis”	Generally fully reversible	Many compounds: e.g., aminoglycosides (245, 246), cephalosporin (354)	
3.1.2. Tubules	Cytoplasmic basophilia	Most likely excessive cell turn-over due to tubulotoxicity	Per se little. May lead to kidney tumors in life-time bioassays	Many compounds (see above)	Relatively frequent in repeat dose studies. May be an early sign of nephrotoxicity

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
	Cytoplasmic inclusions	Storage of many different materials including proteins, compound, pigments, etc.	Probably little, with exception of α_2 -microglobulin tubulopathy, which results in tubular tumors in life-time bioassays	Many compounds α_2 -microglobulin induced by hydrocarbons	Relatively common finding α_2 -microglobulin-associated tumors are of no significance to man (99,355)
	Degeneration/ necrosis	Cytotoxicity	Prolonged tubular toxicity can lead to tubular tumors in life-time bioassays If extensive: kidney failure	Many compounds, e.g., cephalosporines (354), aminoglycosides (245, 246)	Tubular epithelia have a big potential for regeneration and adaption: despite continuous exposure, tubulotoxicity can be transitional
	Hypertrophy	Often age related but occasionally increased after chemical exposure	Increased organ weight	Daunomycin	
	Hyperplasia	Spontaneously or following nephrotoxicity	Preneoplastic lesion, if multilayered (clear or eosinophilic cells)	Induced by many compounds, e.g., cisplatin	

3.1.3. Tubular lumen	Crystals	Consequence of concentrating properties of kidney and/or pH changes	May lead to tubular damage and occlusion	Sulfonamides	
	Dilatation/cysts	For example, tubular stasis following nephrotoxicity	Can reduce kidney function in old animals in the context of other renal diseases	Many nephrotoxins	
3.1.4 Interstitial space	Mineralization	Spontaneous and induced lesion, often associated with increased urinary excretion of Ca	Can reduce kidney function in old animals in the context of other renal diseases	Induced by vitamin D, PTH, Ca glyconate, etc.	In rats, mineralization tends to occur at the corticomedullary junction
3.1.5 Papilla	Necrosis	Reduced renal papillary blood flow because of reduced prostaglandin formation	Can reduce kidney function in old animals in the context of other renal diseases	For example, non-steroidal anti-inflammatory drugs (254, 356)	Mainly in rats and humans
3.2. Urinary bladder	Calculi	Spontaneously and induced	Can lead to cystitis and/or mild hyperplasia and metaplasia of the urothelium	Induced, for example, by sulfonamides, saccharine (162)	Spontaneously frequent in rodents with no consequences
			Can result in bladder cancer in life-time bioassays (93,94,357)		

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
4. Endocrine system (261, 358, 359)					
<i>4.1. Adrenal-cortex</i> (360)					
4.1.1. Regressive changes	Lipidosis (especially zona fasciculata and reticularis)	For example, by inhibition of steroidogenesis with build-up of precursors	Could lead to Addison-like symptoms	Aminogluthethimide and other compounds	Functional impairment rarely seen
	Inclusions of hyaline droplets	Resorption of proteins with formation of vacuoles	Probably none	Starvation, methyl-androstenediol	
	Atrophy	For example, decreased stimulation of aldosterone production in zona glomerulosa	Decreased organ weight Generally reversible	Timolol maleate, captopril, corticosteroids	
	Necrosis	For example, by retrograde embolization of adrenal medullary cells into cortical capillaries	If limited: none	Various compounds including hexadimethrine	

	Cystic degeneration of zona fasciculata/reticularis	Unknown	Unknown	Estrogens, tamoxifen, etc. administered to male rats	
4.1.2. Proliferative changes	Hypertrophy	For example, increased stimulation of aldosterone production in zona glomerulosa	Organ weight increased Can lead to tumors in long-term bioassays	Diuretics, spironolactone	Reversible. Various compounds induce hyperplasia of the adrenal cortex by different mechanisms (361)
4.1.3. Sinuses	Focal nodular hyperplasia Vacuolation of sinusoidal cells	Regenerative reaction to cortical toxicity For example, multilamellated bodies resulting from indigestible membranes in phospholipidosis	Can lead to tumors in long-term bioassays Unknown	Diuretics, spironolactone Tricyclic antidepressants	Sinusoidal cells are part of reticulo-endothelial system with phagocytic function
4.2. Adrenal medulla (360)	Proliferation	Spontaneous and induced, possibly associated with hypercalcemia	Unknown	Induced, for example, by reserpine, retinoids, thiouracil	Especially seen in rats
4.3. Pancreatic islet cells					Infrequently the target of toxicity
4.3.1. Regressive changes	Necrosis Vacuolation	β -Cell toxins β -Cell hypersecretion	Diabetes mellitus Reversible	Alloxan, streptozotocin Cyproheptadiene, cyclizine	

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
4.3.2. Progressive changes	Atrophy	Exhaustion of β -cells: consequence of prolonged stimulation, for example, through lowering of circulating insulin levels	Diabetes mellitus	Growth hormone and others (362)	
	Hypertrophy	Corticosteroids increase blood glucose levels and, therefore, stimulate β -cells	In severe cases, the β -cells become exhausted and atrophic	Corticosteroids	
	Hyperplasia	Prolonged stimulation of β -cells Stimulation of insulin secretion in mice (trophic effect on β -cells)	Can progress to islet cell adenoma Can progress to islet cell adenoma	Any compound which causes hyperglycemia Prolactin	
4.4. Thyroid (363)					
4.4.1. Follicles	Hypertrophy/ hyperplasia	Increase of TSH as a consequence of decreased T3 and T4 levels because of	Increase of organ weight Can progress to follicular adenoma	Thyrostatic agents Compounds, which induce metabolic	The rat is particularly sensitive

		decreased production or increased metabolism		enzymes (133,136,137) or liver mass (364)	
	Atrophy	Decrease of TSH levels	Decreased organ weight, hypothyroidism	Thyroxin (T4), triiodothyronine (T3)	
	Pigmentation	Pigment granules (lipofuscin, melanin, derivatives of test compound) in follicular epithelial cells	Probably none	Minocycline, vincamines	
4.4.2. Parafolli- cular cells	Hyperplasia	Physiological response to increase of blood Ca levels	May progress to adenoma	Vitamin D	
4.5. Parathyroid	Hyperplasia	Physiological response to decrease of blood Ca levels; particularly because of severe nephropathy (induced or spontaneous) with secondary hyperparathyroi- dism	Increased organ weight May progress to adenoma	Age	Frequent in lifetime bioassays

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
4.6. Pituitary	Infarction	Unknown	May lead to atrophy of endocrine organs	Hexadimetrine	Rare
	Vacuolation Hypertrophy/ hyperplasia	Unknown If function, for example, of thyroid is significantly decreased, thyrotrophs become first hypertrophic, then hyperplastic, but may finally become exhausted and atrophic	Unknown Increased organ weight If last stage (exhaustion, atrophy) is not reached, hyperplasia might transform into a tumor	Anticancer drugs Thyrostatics, contraceptives, and neuroleptics producing hyperprolactinemia Calcitonin (207, 365)	Rare
5. Reproductive system					
5.1. Ovary (257)	Oocyte destruction	Cytotoxicity	Sterility if primordial follicles are destroyed (present since birth without regenerative capacity) Can result in atrophy of the whole ovary	Radiation, polycyclic aromatic hydrocarbons, anticancer agents	Primordial (young) follicles are particularly sensitive
	Atretic follicles	Physiological process and increased by hormonal effects and through cytotoxicity	Generally reversible with hormonal compounds May be irreversible with anticancer agents (see above)	Induced by contraceptive steroids, anticancer agents	

5.2. Uterus (257)
5.2.1. Endo-
metrium

Absence of corpora lutea	Disturbance of the estrogenic cycle, which prevents maturation of follicles to corpora lutea	Generally reversible	Contraceptive steroids, in particular, estrogens
Persistence of corpora lutea	Delayed luteolysis through disturbance of the estrogenic cycle	Organ weight increased Generally reversible	Butyrophenone
	Bromocriptine decreases prolactin and prevents normal luteolysis	Organ weight increased Generally reversible	Bromocriptine (188)
Cysts	Retention of fluid in follicles	Organ weight increased	Estrogenic compounds, β -stimulants, bromocriptine
Endometritis	Often associated with squamous metaplasia of glands and/or endo/myometrial hyperplasia	Chronic irritation can result in uterine tumors in life-time bioassays	Estrogenic and progestational compounds
Squamous metaplasia	Response of endometrial epithelium to chronic irritation	Can result in hydrometra (fluid accumulation) and pyometra (pus)	Estrogenic compounds, vitamin A deficiency

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type					
	Lesion	Pathogenesis	Consequence	Examples	Remarks
	Hyperplasia	Response of endometrial glands to prolonged irritation or stimulation	Increased uterine weight	Estrogenic and progestational compounds	
	Decidualization	Unknown	Reversible	Growth hormones	Mainly in rats. Can be mistaken for a uterine neoplasm
	Endometriosis	Endometrium-like tissue (glands and stromal elements) in ectopic (unusual) locations	Probably not highly relevant	Stilbestrol and other estrogenic compounds	Mainly rabbits and mice, where it is also called adenomysis
	Atrophy	Prolonged stimulation of the progesterone type	Marked reduction in uterine weights	Progestational compounds	Mainly in monkeys
5.2.2. Myometrium	Hyperplasia	Physiological response, see also endometrial hyperplasia above	Infertility Increased uterine weight	Butyrophenones Estrogenic and progestational compounds	Mainly in dogs
5.3. Vagina/cervix	Hyperkeratosis/hyperplasia	Increased estrogenic stimulation of the squamous epithelium	Reversible, probably with exception of late stages	Estrogenic compounds	Mainly dogs and monkeys

5.4. <i>Mammary glands</i>	Mucification	Increase of mucus-producing cells in vagina and cervix	Can lead to marked distention of the cervical canal in monkeys	Progestational compounds	
	Atrophy	Mediated through diminished ovarian activity	Reversible, probably with exception of late stages	Progestational compounds	Mainly dogs and monkeys
	Glandular development in immature females or in males/glandular hyperplasia in mature females	Physiological response with proliferation mainly of the ducts and—after prolonged exposure—also of the glands (lobules)	Results, particularly in rats, in glandular secretion with cystic dilation of the ducts	Estrogenic compounds (various species), dopamine antagonists (rats and dogs), progesterone (dogs)	Mammary gland differentiation and hormonal receptor expression are influenced by the diet (366)
	Hyperplastic nodules	Proliferation of glandular and stromal components to various proportions	Can be associated with regressive changes such as sclerosis, etc.	Progestational compounds	
	Atrophy	Lowering of prolactin levels	Reversible, probably with exception of late stages	Bromocriptine (188)	
5.5. <i>Testis</i> (51,54,255,256, 258,260,262)	Necrosis	Cytotoxicity to germ cells at various stages of their development (from spermatogonia to spermatids)	Depletion of spermatogonia will result in sterility with complete atrophy of the seminiferous tubules, so-called	Anticancer agents	Mainly seen in acute to subacute toxicity studies Generally accompanied by cell debris and

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
			Sertoli cell-only tubules		multinucleated germ cells in the epididymis
	Atrophy	Cytotoxicity	Organ weight decreased Sertoli cell-only tubules Sterility	Anticancer agents, estrogenic and progestational compounds. Dopaminergic antagonists and agonists	The most common finding in case of prolonged testicular toxicity
		Spontaneous	Organ weight decreased Foci of Sertolicell-only tubules If not too extensive, testicular function preserved		Foci of atrophic seminiferous tubuli are frequent, particularly in dogs in subcapsular area (367)
	Leydig cell hyperplasia	Inhibition of spermatogenesis increases Leydig cell activity	Organ weight often decreased because of Sertoli cell-only tubules In long-term bioassays after leading to tumors	Estrogenic compounds, dopaminergic antagonists, and agonists	

5.6. <i>Epididymis</i> (51,256)	Spermatic granuloma	Rupture of epididymal ducts	Extravasation of spermatozoa into the interstitium leads to a granulomatous reaction Can be associated with infertility	DL-Ethionine, guanethidine, and other compounds (368)	Can also occur in the testis
5.7. <i>Prostate</i> (86,256)	Atrophy	Physiological and often unspecific reaction	Reduction of organ weight In severe cases, sterility	Estrogenic and progestational compounds	Frequent finding particularly in animals in poor condition
	Hypertrophy/ hyperplasia	Physiological reaction	Organ weight increased	Androgens	
	Squamous metaplasia	Transformation of acinar epithelium	Impaired function	Estrogenic compounds	Particularly in dogs and mice
6. Immune System (41,42,234,249–251, 272,369–370)					
6.1. <i>Thymus</i> ^c					
6.1.1. Lymphatic component	Atrophy	Loss mainly of T-cells with concomitant reduction of the thymal stromal component Physiological involution in adolescence	Organ weight decreased If occurring pre/peri- or early postnatally: T-cell deficiency with reduced resistance to infection	Stress Decreased levels of growth hormone Corticosteroids Anticancer agent, e.g., cyclophosphamide	
6.1.2. Epithelial component	Hyperplasia	Unknown	Can result in cyst formation, etc.	Estrogenic compounds	

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
<i>6.2. Lymph nodes (LN), spleen (S)</i>					
6.2.1. General	Extramedullary hematopoiesis	Physiological phenomenon Sometimes a consequence of systemic diseases (e.g., bone-marrow toxicity) Unspecific sign of toxicity	—	Many compounds	Particularly megakaryocytes (which produce thrombocytes) and granulopoiesis
	Congestion, hemorrhage	Generally agonal	—		Frequent, generally without toxicological significance
	Necrosis	Possibly because of microinfarctions due to erythrocyte agglutination Infarction because of vasoconstriction	Granulomatous partial reparation	Ethyl palmitate, concanavalin A	
6.2.2. Lymphatic components	Decreased cellularity	Decreased number of T-lymphocytes in LN paracortex/in	If not extensive: none If extensive: resistance against infection in reduced	Vasoconstrictive agents Cyclophosphamide (B and partly T cells)	

		periarteriolar sheaths of S and/or of B-lymphocytes in follicles (LNS)		Indomethacin, aspirin (T cells)	
	Atrophy	Depletion of lymphatic tissue	Spleen weight decreased Resistance against infection may be reduced	Corticosteroids at high doses for prolonged periods of time	
	Increased cellularity	LN: for example, through mobilization of lymphocytes from the spleen	—	LN: Heparin, polysaccharide sulfates	
	Germinal center hyperplasia	Physiological stimulation	Often accompanied by plasmacytosis (B cells)	Antigens, infection	
6.2.3. Sinuses	Histiocytosis	Storage of lipids and various kinds of pigments in the LN, into which the application site of xenobiotics (e.g., mesenteric, bronchial, etc.) drains	Can lead to hyperplasia with reduced half-life of erythrocytes and ensuing anemia	Various compounds, including macromolecular polymers	The lining of the sinuses is part of the reticulo- endothelial system (phagocytosis), to which for example, also the hepatic sinuses belong
7. Hematopoietic (252,371) and circulatory system (244,372,273)					
7.1. Bone marrow	Atrophy	Cytotoxicity: proliferating cells are particularly sensitive to toxicity	(Partial) replacement of blood-forming bone marrow by fat tissue Complete bone marrow atrophy is fatal	Anticancer drugs, phenylbutazone (dogs)	Partial atrophy is physiological with age

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
7.2. Cardiovascular system—Heart	Myelofibrosis	Possible consequence of cytotoxicity resulting in proliferation of fibroblasts	Depending on extent. If extensive: bone marrow failure	Irradiation, saponin	Rare
	Hyperplasia	Proliferation of megakaryocytes	Unknown	β -Adrenergic compounds	Dogs only
		Proliferation of monocytes under immunostimulation	Unknown	Muramyl peptide	
		Myeloid hyperplasia as a reaction to inflammatory and/or hemorrhagic conditions	Most likely fully reversible	Secondary to various compounds	
		Formation of germinal centers	Most likely fully reversible	Immunostimulants, infection	
	Necrosis	Cytotoxicity	Multifocal scars, possibly with calcification. If extensive: cardiac insufficiency	Doxorubicin and other anticancer agents (27)	

		Hypoxia by prolonged tachycardia (mainly dogs) or decreased blood pressure	As above	Catecholamines, vaso- and broncho-dilating agents (374)	
	Hypertrophy	Enlargement of myocardial fibers, partly because of increased workload	Increased organ weight	Ca-channel blockers, thyroxine	
7.3. Cardiovascular system—Vessels	Vasculitis	Unclear, possibly vasoconstriction of vasa vasorum and/or insudation of plasma through leaking endothelial cells	Can lead to thrombotic occlusion of the vessel, particularly if evolving to necrotizing vasculitis	Immunostimulators Theobromine, theophylline, dopaminergic vasodilators (240), phosphodiesterase inhibitors (239), and others (377)	Vasculitis can occur spontaneously, particularly in dogs (375,376)
8. Respiratory system (243)					
8.1. Nasal chambers and airways					
8.1.1. Respiratory epithelium	Degeneration/atrophy	Cytotoxicity	Necrosis of the respiratory epithelium may lead to ulcerations and disturbance of the nasal innervation (378)	Irritant chemicals administered by (nasal) inhalation, e.g., formaldehyde	

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
8.1.2. Olfactory epithelium	Hypertrophy/ hyperplasia/ squamous cell metaplasia	Reaction to chronic irritation of respiratory epithelium	Squamous epithelium is more resistant against chemical irritation Probably reversible	Irritant chemicals administered by (nasal) inhalation	
	Goblet cell proliferation	Reaction to subacute irritation of respiratory epithelium	Protective effect of mucus covering the respiratory epithelium	Irritant chemicals administered by (nasal) inhalation	
	Degeneration/ atrophy	Cytotoxicity	Regenerative capacity of the olfactory epithelium is limited, and cell loss might be irreversible	Irritant chemicals administered by (nasal) inhalation Acetaminophen, phenacetin	3-Methylindole administered systemically can damage the olfactory epithelium in mice
8.2. Lung parenchyma	Congestion, alveolar hemorrhage	Generally an agonal finding, but can accompany severe toxic lung lesions	If toxicity-related: disturbance of gas exchange between alveoli and blood vessels If agonal: without significance	Bleomycin, paraquat, hyperbaric oxygen, cyclophospha- mide	
	Edema, exudation of fibrin, etc	Often secondary to toxic lung injury or pneumonia	Increased organ weight Can be associated with fibrin deposits on the alveolar wall, hyaline	Bleomycin, paraquat, hyperbaric oxygen,	With unexpected animal deaths in gavage studies, instillation of test

		membranes. Can be resolved or lead to intra-alveolar and interstitial fibrosis	cyclophosphamide	solution by mistake into the trachea to be considered
		In severe cases necrotizing pneumonitis with respiratory insufficiency		
Diffuse, unspecified, alveolar injury	Cytotoxicity mainly of type 1 pneumocytes	May lead to epithelialization (cuboid type 2 pneumocytes, which later transform into type 1 pneumocytes; this signals recovery)	Bleomycin, paraquat, hyperbaric oxygen, cyclophosphamide	
		Gas exchange likely to be impaired		
Adenomatosis	Adenomatous hyperplasia of type 2 pneumocytes	If not generalized: no significant consequences	Dimethylhydrogen phosphite	
Intracellular storage of various substances	Essentially in type 2 pneumocytes, e.g., phospholipids Dust particles	Even with extensive storage disease generally no clinical signs	Amphiphilic drugs given systemically (phospholipidosis) (331) Inhalation, for example, of quartz dust	Storage can also occur in macrophages present in alveolar septa, especially in subpleural, peribronchiolar, and perivascular regions

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
	Chronic inflammatory foci	Accidental infection	None, if the number of inflammatory foci is not excessive	Spontaneous Exacerbation with immunosuppressive agents	The number of infectious foci is a measure of the quality of the test animals
Perivascular granulomas	Embolization after intravascular injection of crystalline material, hair, and skin fragments	Embolization leads to thrombosis, and perivascular granulomas and can evolve into focal interstitial pneumonia	Compounds, which are difficult to dissolve Long-term i.v. studies (skin and hair fragments introduced into vessels)		
9. Nervous system (52,266,267,379,380)					
9.1. General	Vacuolar encephalopathy	Unknown	Unknown	Incidence and severity can be increased by treatment with certain compounds	Vacuolation in the nervous system is often an artifact
9.2. Nervous tissue					
9.2.1. Neural cell body	Degeneration and necrosis	Cytotoxicity	Damage to the cell body usually results in degeneration of axons and dendrites, as well as the myelin sheath	Mercury, tri/tetramethyl, lead	Rare in toxicity studies

9.2.2. Axons	Degeneration (with fragmentation and variation of axonal thickness)	Excessive stimulation of neurons with exhaustion Interference with vital axonal processes. Probably often associated with disturbed transport of vital nutrients and components (e.g., proteins) from the cell body to the peripheral axon	Loss of innervation Axonopathy is followed by secondary demyelination and by chromatolysis, but generally not followed of death of the cell body After discontinuation of exposure some recovery is possible	Excitatory amines, e.g., glutamate and derivatives Chloramphenicol, diphenylhydantoin, isoniazid, nitrofurantoin, vincristine, organophosphates	Axonopathy occurs mostly in distal axons
9.2.3. Myelin sheath	Myelin vacuolation and degradation	Direct action on myelin, on myelin producing Schwann cell or on oligodendrocyte metabolism	After discontinuation of exposure some recovery is possible	Hexachlorophene, isoniazid, triethyl tin, actinomycin D	Long myelinated axons are particularly vulnerable
9.3. Glia	“Activation” including proliferation around lesions	Secondary reaction to toxic injury to neurons	Phagocytosis of neuronal debris etc.	Hexachlorophene isoniazid, triethyl tin, actinomycin	Primary toxic changes to glial cells are uncommon
9.4. Choroid plexus	Vacuolation	Unknown	Unknown	Disobutamide, chloroquine	

10. Sensory System

10.1. Eye (381–383)

10.1.1. Cornea	Keratitis/ conjunctivitis
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Generally following topical application	Chronic irritation can lead to	Any irritant chemical
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(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type					
	Lesion	Pathogenesis	Consequence	Examples	Remarks
	Ulceration	or irritant chemicals or accidentally (e.g., fighting among animals)	neovascularization of cornea, which is normally devoid of vessels Ulceration might lead to perforation of cornea with loss of eye		
10.1.2. Uveal tract	Corneal vacuolation Uveitis, vacuolation, etc.	Phospholipidosis Unknown	Impairment of vision Unknown	Amphiphilic drugs Seen occasional with a variety of chemicals	
10.1.3. Lens	Cataracts (turbidity)	Osmotic stress	Impairment of vision	High amounts of lactose, xylose, glucose, certain drugs (384)	Also known to occur in diabetes mellitus
		Secondary to keratitis, iridocyclitis, etc.	Impairment of vision	Any irritant chemical	
10.1.4. Retina	Retinal degeneration	Direct effects on photoreceptor and secondary to degeneration of the optic nerve	Debris of the degenerating photoreceptors are phagocytosed by the retinal pigment epithelium	Light, propionic acid derivatives, hexachlorophene	The retina, particularly of albino animals, is very light sensitive (385)

	Degeneration of the tapetum lucidum	Chelation of Zn in tapetum lucidum	Most likely impairment particularly of night vision	Ethambutol, dithizone	Dogs only
10.1.5. Optic nerve	Papillary edema	Cytotoxicity	Can result in retinal atrophy	Methanol	
10.2. <i>Lachrymal glands</i>	Decreased lachrymal secretion, partly with some inflammation and cell necrosis	Partly unknown, partly pharmacological effect (e.g., with anticholinergic drugs)	Leads to keratoconjunctivitis sicca (see also above under keratitis)	Sulfonamides, 5-aminosalicylic acid, anticholinergic drugs	Mainly in dogs
	Atrophy	Unclear, possibly autoimmune disease	Dry eye syndrome with corneal ulceration and possibly perforation	β -Adrenergic receptor blockers	
10.3. <i>Harderian gland</i>	Chromodacryor- rhea (pigmented tears)	Normal porphyrin excretion in female animals	—	Increased in inhalation studies with irritant chemicals	No Harderian glands in man
10.4. <i>Ear</i>	Degeneration of parts of the organ of Corti	Cytotoxicity of hair cells	Impairment or loss of hearing	Aminoglycoside antibiotics, quinine	Because of its location in the cranial bones, the investigation of the ear is rather difficult and not done routinely

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
	Edema of the stria vascularis	Possibly by swelling of the vascular endothelium and ensuing hypoperfusion	Lowers the potassium level in the endolymph and, therefore, the function of the organ of Corti	Salicylates, furosemide	
11. Skin, musculoskeletal system					
<i>11.1. Skin</i>	Inflammation	Cytotoxicity of topically applied chemicals	Can lead to ulceration	Methylsalicylate, benzalkonium	
	Hyperplasia	Chronic irritation by topically applied chemicals	Increase of resistance against irritation	Non-steroidal anti-inflammatory drugs	
	Alopecia	Loss of hair/fur related to endocrinopathy	Can be associated with dermatosis-like skin changes	Estrogens, corticosteroids	
<i>11.2. Locomotor muscles (386)</i>	Atrophy of muscle fibers	Malnutrition (e.g., because of severe toxicity) Nerve damage (e.g., because of neurotoxicity) Inactivity associated with painful alterations of skeletal system	If extensive: impairment of locomotor activity	Any toxin given at high enough dose See nervous system	Overall, locomotor muscles are rarely affected by toxicity

		Cytotoxicity	See above	Emetine	
		Endocrine regulation, which favors replacement of myocytes with fat cells	See above	Coritcosteroids	Monkeys
	Necrosis	Toxicity at the site of intramuscular injection or after systemic application	Followed by regeneration of myocytes or by (partial) scar formation If extensive: impairment of locomotor activity	Any irritant compound given intramuscularly Some lipid-lowering drugs given systemically (387) Adriamycin	
11.3. Skeletal system ^d					
11.3.1. Joints	Arthropathy	Cytotoxicity of chondroblasts	Limits locomotor activity Can be associated with synovial hyperplasia	Alkyl pyridone carboxylic acid and derivatives, quinolone antibiotics	
	Arthritis	Cytotoxicity of chondroblasts, immunological effects, etc.	As above	6-Sulfonyl-aminoindazole	Rats
11.3.2. Bones	Osteoarthritis	Age-related, but may be aggravated by administration of sex hormones	As above	Spontaneously Sex hormone	Mice

(Continued)

Table 7 Typical Non-neoplastic Toxicological Lesions^a (*Continued*)

Organ: Cell type, lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
	Osteomalacia	Inadequate calcification of bone matrix and wide osteoid seams because of lack of Ca	Can lead to deformation of bones	Vitamin D deficiency, tetracycline, bisphosphonates	
	Osteoporosis	Decreased osteoblastic activity	Can lead to fractures	Estrogens	
		Increased osteoclastic activity	As above	Retinoic acid	
		Decreased absorption of intestinal Ca	As above	Corticosteroids	
		Renal loss of Ca	As above	Kidney failure with secondary hyperparathyroidism	
	Proliferations	Bone-marrow injury, followed by local inflammation and proliferation of endostium and myeloid stroma	Unknown	Anticancer agents (388)	

^aFor general textbook and overviews see [Refs. 70–72, 126–128, 327–330](#).

^bToxicologically relevant non-neoplastic changes of the kidney glomeruli are rare. Glomerulonephrosis occurs as age-related spontaneous disease.

^cThe thymus is a so-called lympho-epithelial organ.

^dOverall, skeletal system infrequently affected by toxicity.

The following reactions are *difficult to elicit* in test animals used in toxicity tests:

- Immunological reactions, including anaphylaxis and eczema
- Arteriosclerosis. Mild atherosclerotic lesions can be induced by excessively high lipid diets

Furthermore, idiosyncratic drug reactions in humans are likewise generally unpredictable by toxicological investigations. Such reactions are rare and often based on a particular sensitivity of the affected individual, such as in the case of a rare metabolic peculiarity.

Lesions associated with impaired or changed organ function can also affect other organs resulting in “syndromes” such as the hepato–renal syndrome (132), thyroid changes associated with liver hypertrophy (133–137), changes in bones (138) and parathyroids (139) associated with renal disease or the association of hypoxemic lung pathology with adrenal medullary proliferations related to stress (140). Some of these syndromes will be explained in more detail below (Sec. 5.2).

5. TYPICAL NEOPLASTIC CHANGES SEEN IN LIFE-TIME BIOASSAYS

5.1. Introduction

The most important lesions in life-time rodent bioassays are tissue proliferations: hyperplasia, benign tumors, and malignant tumors. The *biological behavior* of these lesions is often not well understood, as—in contrast to human pathology—no biopsies and follow-ups are available. This also renders the establishment of the cause of death difficult (76,141). Histopathological diagnosis of proliferative lesions in rodents is partly based on assumptions and agreement among pathologists, including size criteria to label a tumor as benign or malignant (12). Therefore, standardization of diagnostic criteria is particularly important and the method should be referenced in the report (63,64,78). Deviation from standardized diagnostic criteria is permissible, but must be justified and defined.

Analysis and interpretation of life-time rodent bioassays is among the most *demanding* tasks a pathologist must face (142,143). The development of a compound can be jeopardized by adverse pathological findings just before introduction to the market. On one hand, it is good to remember that there are many more animal carcinogens than human carcinogens (144,145). On the other hand, toxicologists and toxicologic pathologists have the responsibility to make sure that humans and the environment are not exposed to real carcinogens. The diagnostic quality depends on the qualifications and ability of the pathologist, which necessitates many years of postgraduate training in pathology (146). Board certification (US, EU,

IATP) or registration for toxicologic pathology (EU, J) is another hallmark of qualification in pathology (75,147).

As life-time bioassays are large studies with 500 and more animals, each with some 60 organs, and as these studies are generally performed towards the very end of development, they are *time-critical*. Nevertheless, it is advised that only one pathologist should read the histological slides. If, for deadline reasons, two pathologists must share the task, then splitting by males and females may be considered. Splitting by dose groups should be avoided. All the slides from one animal are generally read consecutively, as the various lesions are interdependent and often have biologically significant effects on the overall condition of the animal (as mentioned above in Sec. 4.1).

Sometimes, *transgenic mice* are used for carcinogenicity testing. Examples include the p53+/-, TgHras2 and XPA transgenic mice, or neonatal mice (148–160). The theoretical advantage of these models is the earlier appearance of tumors, which allows shortening of the experimental duration. So far these models have not gained full acceptance, partly because it was recognized that the genetic construct is unstable and can be lost. The technology underlying transgenic models and the role of transgenic animals in preclinical drug development is the focus of [Chapter 3](#).

Because of the complexity of tumor diagnosis and since information about diagnostic criteria of various tumor types is readily available in the scientific literature (65–72) and on the internet (65,66), this section is limited to a general description of the pathogenesis of tumors, their histological appearance, as well as of their precursor lesions.

5.2. Pathogenesis of Tumors

The basic pathways of tumor formation include:

- *Genetic alterations* (mutagenicity, chromosomal anomalies, etc.). With today's genotoxicity testing, genotoxic chemicals are rarely tested in life-time bioassays. However, the relatively large number of spontaneous tumors may well have a genetic component, resulting from inbreeding and/or spontaneous genetic alterations.
- *Epigenetic alterations* (nongenotoxic mechanisms) are now more likely to account for tumor induction in life-time bioassays (161). As mentioned earlier, essentially all conditions with increased cell proliferation are likely to result in increased tumor incidences long-term. Increased cell proliferation is present in the so-called tumor precursor lesions, particularly in the following conditions:
- *Chronic tissue irritation*. Test substances may crystallize and form aggregates with proteins and lipids from body fluids in the excretory system. One such example is saccharine which leads to urinary bladder carcinoma if fed for life-time to male

rats (93,162). Similarly, solid state carcinogenicity occurs mainly in rodents; for example, at subcutaneous injection sites in rats (163) or at implantation sites of microchips in mice as used for animal identification (164,165). The latter connective tissue tumors are of little relevance in man, whose connective tissue is much less sensitive to chronic irritation.

- *Increased physiological stimulation of cells* leading to cellular hypertrophy (increase in size, generally with proliferation of cellular organelles) and hyperplasia (increase in cell number). It is well known that hepatomegaly seen with liver enzyme inducers in mice during subchronic toxicity studies is often an early indicator for liver tumors in the mouse life-time bioassays (166). Liver enzyme inducers can lead not only to liver tumors because of long-term stimulation of hepatocytes, but also to thyroid tumors because of increased T3 metabolism and the consequent TSH increase with chronic stimulation of the thyroid follicular cells (133,136,137).

Both findings are of no relevance to man.

5.3. Species-Specificity of Tumor Formation

The influence of the species is reflected in the wide variation of incidences of spontaneous pathology findings among different rodent *species*. Mice have a high incidence of spontaneous tumors particularly in lung, liver and also in Harderian and adrenal glands, the hematopoietic system and ovaries (167–176). Rats have a high incidence of mammary gland and pituitary tumors (174,177,178). Rats and mice may also differ in their response to life-time exposure to chemical substances, but in most cases, data from mice and rat life-time bioassays are in agreement (179).

There are also marked differences in tumor incidences between *different strains* of the same species. Examples include the following:

- Leydig cell tumors: 88–96% in F344 rats, below 10% in Sprague–Dawley (SD) rats, and 1–2% in Long–Evans rats (180)
- Mononuclear cell leukemia: 20% in F344 rats, rare in SD rats (181)
- Mammary gland tumors, the incidence of which varies widely between different strains, possibly due to endocrine differences (182) and viral infections (125).

Significant *intra-strain differences* exist also between animals from different breeders (183). Gene expression can vary in genetically homogenous animal populations, leading to differences within that population (184).

Formation of tumors in endocrine organs resulting from disturbance of *hormonal balance* is particularly frequent in life-time rodent bioassays. However, their occurrence is of little significance to man, mainly as the

physiology of rodents is quite different from man in several respects (185). Particularly, the endocrine regulation of rodents is special and easily disturbed. For example, rats lack high-affinity thyroxin-binding globulin (186,187); the estrogen/progesterone ratio is 1:100–200 in rats, but 1:1 in women; reproductive senescence means progesterone dominance in old rats, while in women the sex hormone production just decreases (188); prolactin has a trophic effect on the mammary gland of rats, while it maintains lactation in women (189); rat, but not human Leydig cells react with tumor formation following long-term stimulation by high LH levels (180).

Rodents have additional *anatomical particularities*. The following organs are not found in man: forestomach, Harderian gland (eye region), Zymbal's gland (ear), and preputial/clitoral gland. Tumors occurring exclusively in these organs are often regarded as not relevant for man (190,191), although tumors in the forestomach might indicate, for example, a risk for esophageal tumors in man and the similarity of Zymbal's and preputial/clitoral glands to human sebaceous glands must be kept in mind.

Further differences in the reaction of rodents and man to toxic injury, particularly also with regard to tumor formation, may be based on *pharmacokinetic differences* (135,192,193). Therefore, among other aspects the following need clarification: which metabolites are formed and could they be genotoxic (e.g., higher amount of genotoxic tamoxifen metabolites in test animals) (194)? Does the compound have a special affinity for a particular organ? How is the compound excreted? (See also [Chapters 4,5,8](#)).

5.4. Histopathogenesis of Tumors

Tumors, which are induced by chemicals, are generally morphologically not different from spontaneous tumors. The development of tumors is often a continuous process, though not all stages have to occur in a textbook-like manner (see [Table 8](#)). Because of their continuous *nature*, the various stages often overlap, and classification into one or the other stage is arbitrary (e.g., based on the size of the lesion). This holds true in particular for the distinction between precursor lesion (hyperplasia) and benign tumor of the endocrine organs. Leydig cell hyperplasia in the testis of more than a certain diameter is called an adenoma, though this “tumor” could possibly still regress (and therefore not fulfill the criterion of a tumor), if the endocrine regulation would revert to normal (normal LH levels). In contrast to human pathology, animal pathology generally does not have the benefit of a follow-up of the individual (biopsy, surgery with histological assessment, after surgery, etc.).

Similarly, and for the same reasons as given above, the distinction between *benign and malignant tumor* is also not always clear-cut. Metastases are not frequently seen in life-time bioassays. In view of the small size of the animals, metastases may also be missed macroscopically with the consequence that the affected portion of the organ is not sampled for histology.

Table 8 Tumor Formulation: Histopathological Sequence

Type of lesion		Definition	Underlying reason	Example
<i>Precursor lesions</i> Alterations are reversible and do not compress or infiltrate surrounding tissue	(Hypertrophy)	Increase in cell size, often associated with and difficult to distinguish from hyperplasia	Proliferation, for example, of SER	Barbiturates, which induce hepatic P450 cytochrome drug-metabolizing enzymes
	Metaplasia	Transformation of cells into a type generally not seen at this location	For example, replacement of respiratory epithelium by squamous cell epithelium	Chronic irritation during inhalation studies, for example, by formalin
	Hyperplasia	Increase in cell number, often associated with hypertrophy	Chronic stimulation of cells	Chronic irritation and inflammation at subcutaneous injection site
<i>Tumor</i> Not reversible. At least some autonomous growth with compression and/or invasion of surrounding tissue	Benign	Tumor remains delimited by connective tissue capsule. Does generally not infiltrate surrounding tissue	Expansion of (spontaneously) initiated cells (mutagenic defect) by mitogenic stimulus	Thyrostatic agents induce adenomas of thyroid follicular cells
	Malignant	Tumor infiltrates surrounding tissue and often also blood vessels, which results in metastases to distant organs	Expansion of (spontaneously) initiated cells (mutagenic defect) by mitogenic stimulus	Anticancer agents

5.5. Tumor Types

Tumors are classified according to

- Organ
- Cell-type
- Biological behavior: benign, malignant, but see also above ([Sec. 5.4](#))
- Degree of differentiation: the less differentiated, the more aggressive they generally are
- Growth: with increasing malignancy, generally the velocity of growth also increases

An overview of common tumor types found in life-time rodent bioassays is given in [Table 9](#).

Basically, all type of cells and all tissues can develop into tumors, both of the benign and the malignant type. Species, strain, breeder, and dose and route of application of the test substance influence the organ-specific tumor incidence (195). Overall, there are no marked differences in the distribution of tumors induced by mutagens and nonmutagens (196,197), although differences were described for some substances, such as chlormethine (198,199). Epigenetic carcinogens usually increase tumor incidences in organs with a high spontaneous rate of tumors, and lead to neoplasms with similar histology as their spontaneous counterparts (106). This is further supported by data published in the Physician's Desk Reference (144), which cites the most frequent target organs in rats and mice as:

- Rats: thyroid, liver, testis, mammary gland, adrenal, and pituitary
- Mice: liver, lung, mammary gland, bone marrow, and ovary.

Small, but statistically significant, increases in the above tumor incidences are often regarded as less relevant than the appearance of new tumor types. This is the case with tumors in livers (200), particularly in mice livers (201,202).

5.6. Pooling of Incidences of Similar Tumors

To increase statistical power of rare observations, one often has to combine the incidences of similar tumors; for example, tumors with similar histogenesis such as benign and malignant tumors of the same cell type in the same organ/organ system (e.g. liver adenoma and carcinoma; leukemia and lymphoma). At times, it is reasonable to combine tumors also with precursor lesions (e.g., liver adenomas and carcinomas with hyperplasia/metaplasia). The rationale for combining proliferative lesions is as follows:

- Distinction among hyperplasia, benign tumors and malignant tumors is often arbitrary, as explained earlier.

Table 9 Tumor Types seen Particularly in Life-Time Rodent Bioassays

Cell of origin	Cell/tissue	Benign tumor	Malignant tumor
Epithelium	Squamous	Squamous cell papilloma	Squamous cell carcinoma
	Transitional	Transitional cell papilloma	Transitional cell carcinoma
	Glandular		
	Liver cell	Hepatocellular adenoma	Hepatocellular carcinoma
Connective tissue	Follicular cell (thyroid)	Follicular adenoma	Follicular adenocarcinoma
	Fibroblasts, fibrocytes	Fibroma	Fibrosarcoma
	Cartilage	Chondroma	Chondrosarcoma
	Bone	Osteoma	Osteosarcoma
Muscle	Fat	Lipoma	Liposarcoma
	Smooth muscle	Leiomyoma	Leiomyosarcoma
	Skeletal muscle	Rhabdomyoma	Rhabdomyosarcoma
	Cardiac muscle	Rhabdomyoma	Rhabdomyosarcoma
Neural tissue	Nerve sheath	Schwannoma	Malignant schwannoma
	Glial cells	Glioma	Malignant glioma
	Astrocytes	Astrocytoma	Malignant astrocytoma
	Embryonic cells	—	Medulloblastoma
Endothelium	Lymph vessels	Lymphangioma	Lymphangiosarcoma
	Blood vessels	Hemangioma	Hemangiosarcoma
Hematopoietic	Bone marrow	—	Leukemia
Lympho-reticular	Lymph nodes	—	Lymphosarcoma

For histopathological atlases of rodent tumors, see [Refs. 65–69, 389, 390](#). For hamsters, consult the following Refs. 391,392.

- As generally only one section per lesion is examined, the most malignant portion of a neoplasm might not be available.
- One cell type can give rise to different types of proliferative lesions through differentiation (e.g., epithelial basal cells), dedifferentiation (generally associated with increased malignancy) and show various growth patterns (e.g., mammary gland and endocrine neoplasms: cystic, papillary, and solid growth patterns). Progression over time can change the histological appearance of proliferative lesions significantly.

- For risk assessment, the distinction between benign and malignant generally is not of great importance, with exception of rare cases, when a substance only increases the aggressiveness of tumors.

The following proliferative lesions can generally be combined in life-time bioassays (203):

- Neoplasms in the respiratory tract or in the peripheral lung, but generally not from both locations together
- Squamous cell neoplasia of the upper alimentary tract including forestomach
- Epithelial neoplasm of the small intestine or the large intestine and—depending on the type of proliferative lesions—also of small and large intestine combined
- Smooth muscle neoplasia body-wide with exception of those of the GI tract and reproductive tract, which must be evaluated separately

The types of proliferative lesions which can be combined have often to be determined on a case-by-case basis (204).

6. INVESTIGATION OF UNCLEAR PATHOLOGICAL FINDINGS

6.1. Additional Investigations of Available Samples

If unclear histopathological findings are detected, additional investigations might be warranted (205). They can sometimes be performed on samples already available from the study in question, or from previously conducted toxicity studies (206). Examples for possible investigations include:

- *Blood samples for hormones* (207,208), particularly for findings in endocrine organs. As already mentioned, hormone levels provide important insights into the trophic effect on a particular endocrine regulated organ. Increased or decreased stimulation of an organ is associated with hypertrophy/hyperplasia or—conversely—atrophy of the organ in question. Hyperplasia is often associated with tumor formation in that particular organ in life-time rodent bioassays.
- *Blood or tissue samples for gene or mRNA expression or for marker proteins* or protein patterns by proteomics (209). Such investigations have become fashionable in the past few years. However, the future will have to show to what extent they are actually useful.
- *Tissue samples for investigations by EM or by immunohistochemical methods*. Applications include identification of the proliferative cell type (16,210), measurement of cell proliferation (by PCNA) (57,211) or apoptosis (212). Even with formalin-fixed tissue, EM investigation may show important subcellular details which help

to confirm or reject a pathogenetic mechanism. The presence of a phospholipidosis can easily be proved by EM investigations on formalin-fixed tissue. Molecular epitopes are astonishingly stable on fixed tissue and can be demonstrated immunohistochemically even after many years in wet tissue and paraffin-embedded tissue. It is always worthwhile to test such material, as long as negative and positive controls are run simultaneously.

- Morphometry is important to prove or disprove numerical or volume changes in tissues and particularly to establish a no-effect level (13).

6.2. Tailor-made Mechanistic Studies

Additional studies may be envisaged, particularly for the following purposes:

- *To repeat any of the above investigations on properly sampled and prepared tissue*; for example after fixation with glutaraldehyde by EM (206) or more detailed cell kinetic studies (proliferation, single cell necrosis, etc.) (213).
- *Investigation of early findings and their development over time* (e.g., by sequential necropsies in a time-course study)
- *Reversibility studies*, with necropsies also after a treatment-free period
- *Mechanistic studies* to test hypotheses that cannot be tested with enough certainty on material already available
- *Special in vitro studies* with cell or tissue cultures, organ slices or the perfused target organ itself to investigate metabolism (214), effects on subcellular organelles (see also [Sec. 3.2](#)) or on gene expression (22,26–31), or dose–effect relationships at subcellular or molecular levels (215). Receptor studies (216,217) can be very helpful, while studies of intercellular communication (218) and initiation–promotion assays (219) may not contribute much.

7. INTERPRETATION OF PATHOLOGICAL FINDINGS

Interpretation of toxicological findings always has to start with a number of questions, such as:

- Is the study technically valid?
- Is the model valid?
- Is there indeed an adverse effect? Could the adverse effect be species-specific?
- Were there other relevant findings?

- What is the pathogenesis of the lesion and what is known about other substances of the same class?

Each of these questions is described below

7.1. Is the Study Technically Valid?

There are a number of reasons, why a study can be flawed. Three selected aspects are mentioned below.

- One key factor is *dose selection*, particularly in rodent life-time bioassays (220–223). The high dose must induce minimal signs of toxicity, but should not significantly interfere with the growth of test animals or exert overt toxicity (224–226)
- Was the *test substance* analytically characterized according to current standards (227)? How pure was it and what were the impurities? Could an impurity, such as a by- or degradation-product, contaminant (227) or in case of a racemate the pharmacologically less effective enantiomere (228), be responsible for the toxic response seen?
- Were the *technical pathology procedures* appropriate? This includes, for example, consistent necropsy, sampling, and histo-technical procedures (44,46). Tissue accountability is an important factor to judge the quality and validity of a life-time bioassay. The quality of study conduct depends significantly on the personnel involved and particularly on the experience of the study director and study pathologist (75,147).

7.2. Is the Model Valid?

In particular, is the *species* tested representative for man with regard to anatomy and physiology, absorption, distribution, metabolism and excretion of the test compound or to the pharmacodynamic action of drugs (135,229–231)? However, for long-term bioassays, there may not be valid alternatives. For example, albino rodents are not an appropriate model if toxicity studies in pigmented animals (dogs, minipigs, etc.) have shown a particular affinity of the test compound to the pigmented cells. Is the breeder, who supplied the test animals, reliable (184) and the *quality of animals* acceptable with regard to latent infections (123–125)? Was the husbandry good and the diet nutritionally sufficient (232) and without contamination?

To *extrapolate findings* in test animals to human beings a number of conditions have to be fulfilled:

1. Comparable systemic exposure with regard to
 - a. test compound and its metabolite
 - b. distribution plasma levels, and elimination half-life in vivo

If adverse effects in test animals occur only at plasma levels that are at least 100 times more than those achieved in man, there is a good chance that the effects are not clinically relevant.

2. The finding must occur in an organ with similar physiology and anatomy as in man. Rodents have anatomical particularities, as discussed in Sec. 5.3. Aspects of extrapolation of high dose animal findings to risk at low dose exposure in humans are discussed in Sec. 7.6.

7.3. Is There Indeed an Adverse Effect?

Pathologists compare the incidence, severity, and time of onset (as far as possible) of lesions in dosed and control animals. Occasionally, control animals might appear to have an unusually low (or high) incidence of a particular lesion. This can be troublesome, particularly when it comes to the evaluation of long-term rodent bioassays, mainly for the following reason: overtly genotoxic drugs are recognized early in development and, with the exception of certain classes such as antineoplastic drugs, they are not further developed. Drugs, which tested negatively in short-term mutagenicity and genotoxicity assays, may still have a *weak carcinogenic potential* in life-time rodent bioassays, usually due to epigenetic mechanisms. The variability of spontaneous rodent tumor incidences (64) and the large number of statistical tests performed on tumor data from life-time rodent bioassays often result in equivocal results (82). If small increases of tumor incidences achieve statistical significance, then one needs to differentiate between a biologically nonrelevant finding and a true carcinogenic effect. This is particularly difficult in the case of a rare tumor or with a slightly increased incidence of a common tumor in high dose groups. Historical control data can be invaluable in these circumstances. For a discussion regarding the use of historical data, please refer to [Sec. 2.4.3](#).

Crucial and not infrequently controversial is the distinction between adverse effects and e.g. exaggerated pharmacological or adaptive effects (233–235). This is addressed in Sec. 7.5 below.

7.4. Were There Other Relevant Findings?

Pathological findings must be *correlated* with in-life observations, clinical chemistry findings, and any other findings, such as results from pharmacological or mechanistic studies. There must be close interaction among the pathologist, the study director and any other scientist involved in the study (or anyone having performed or is planning to do further studies with the same compound).

Clinical chemistry findings of biochemical toxicity must not necessarily have a histopathological correlate (structural toxicity), but may help the pathologist to correctly interpret histopathological observations. Since investigations of clinical chemistry are conducted repeatedly during a

toxicity study, they also provide information on the time course of a target organ lesion.

Findings of other studies, including mechanistic studies, can be very helpful to interpret pathological findings. If new findings were noticed in longer-term studies, re-examination of that particular organ from shorter-term studies is a must. Furthermore, published data and data obtained from regulatory agency (e.g., under the Freedom of Information Act), are additional sources of information. Knowledge of the mechanism of action helps considerably, especially in the assessment of tumorigenic findings in life-time rodent bioassays (135,236–238).

7.5. What Is the Pathogenesis of the Lesion and What Is Known About Other Substances of the Same Class?

What is the pharmacological action of the compound? Is the toxicity just a consequence of exaggerated pharmacological action or is it an intrinsic part of pharmacological action? To answer these questions, it is always worthwhile to review the published literature or to obtain information from regulators about similar compounds.

7.5.1. Toxic lesions

The following is a summary of some important toxic effects generally seen with particular classes of drugs:

- Arteritis with phosphodiesterase inhibitors (239), some dopaminergic compounds (240), some endothelial antagonists (241) and other compounds (242)
- Phospholipidosis with amphiphilic drugs, such as tricyclic antidepressants (243)
- Cardiotoxicity with anticancer drugs of the anthracyclic antibiotic type (27,244)
- Nephrotoxicity with aminoglycosides and other antibiotics (245–248)
- Atrophy of rapidly proliferating tissues, in particular of bone marrow, lymphoid organs, seminiferous tubules with anticancer agents (249–252)
- Ulcers in the intestinal tract and papillary necrosis in kidneys, the latter particularly in dogs with non-steroidal anti-inflammatory drugs (NSAID): (247,248,253,254)
- Seminiferous tubule atrophy with estrogenic compounds (54,255,256,258–263)
- Pseudopregnancy with persistence of corpora lutea with progestational and dopaminergic compounds, e.g. with neuroleptics (261)
- Thyroid insufficiency with sulfonamides (261)

- Hepatomegaly because of proliferation e.g. with phenobarbital (14,101,235) or because of peroxisome proliferation e.g. with certain hypolipemic drugs (97,103,104,264,265)
- Neuronal toxicity with organophosphates (266,267)
- Myocardial infarction with vasoactive substances (244)
- Cardiopathy with positive inotropic agents in dogs (244)

7.5.2. Carcinogenicity

There are two basic types of tumorigenesis namely genetic and epigenetic (97,268–270)

There are a number of compounds on the market, which are carcinogenic in animals and humans because of their mode of action. Examples include alkylating anticancer drugs (142–144) and immunosuppressive drugs (271–273). For this type of compound, extrapolation from test animals to man is regarded as meaningful.

However, several classes of drugs are known to be animal carcinogens but are regarded as safe for man (144), as discussed previously (i.e., precursor lesions). These compounds belong to the class of epigenetic carcinogens, which are classified below.

Carcinogenic class effects (epigenetic carcinogenicity):

- Compounds inducing *drug metabolizing enzymes* (best known representative: phenobarbitone) (101,235)
- *Peroxisome* proliferators such as hypolipemic drugs, including the clofibrates (104,264,274)
- *Hormones* and other compounds with some endocrine (side) effects: hyperplastic and neoplastic changes are often seen following hormonal and other receptor-mediated stimulation (261). Bromocriptine and mesulergine, both dopaminergic compounds that lower prolactin levels, significantly decrease the incidence of endocrine tumors but increase that of uterine tumors (bromocriptine: (188)) and of Leydig cell tumors (mesulergine: (208)). It is also known that tumors of endocrine organs are often associated with tumors of the adenohypophysis.
- Compounds which—by their intended pharmacological action—*block the normal function of an endocrine organ* or system, often lead to tumors in this organ or system (261). For example, H₂-blockers lead to “carcinoids” in the rat stomach (275–277) and antithyroid agents result in thyroid tumors (136,137). These tumors are without relevance to man.
- Similar findings are reported with other compounds, which *disrupt endocrine function* through an adverse action: for example, sulfonamides interfere with thyroid function and lead to thyroid tumors only in rodents (136,137,278).

- β 2-agonists (e.g., salbutamol or terbutaline) cause hyperplasia of salivary glands and mesovarian leiomyomas in rats, the pathogenesis of which is not well understood (279). Their occurrence can be suppressed by simultaneous administration of a β -blocker, such as propranolol.

The list of animal and human carcinogens is regularly updated in the IARC monographs (142,143,280).

7.6. Extrapolation of High Dose Findings in Animals to Low Doses as Typically Applied to Man

Extrapolation of high dose findings in animals to estimate the risk of side effects and toxicity at low dose exposure of man is an old and still controversial subject (281–286, [Chapters 2, 4, 12](#)).

- Effects produced by *genotoxic and mutagenic compounds* in animals are generally assumed to be relevant for man. In addition, such effects do not have a threshold, below which exposure is considered safe (287).
- General *toxicity and epigenetic tumorigenicity* are considered to have a threshold and therefore, many rodent carcinogens are considered safe for man (91,144,288,289).

For overall safety assessment, often only a *weight of evidence* approach is possible (236) and the relevance of toxic or tumor findings has to be evaluated on a case-by-case basis (290,291). One also notes a considerable evolvement over time, particularly regarding the assessment of toxicity and tumor findings, which has become less schematic and more pathogenesis-oriented (292,293).

On the other hand, much discussion is ongoing regarding the *shape of dose–response curves*. The possibility of, for example bell-shaped dose–response curves cannot be discounted a priori (294,295). Compounds have various effects, which could at least theoretically counteract each other. For instance, a carcinogen might induce fewer tumors at higher doses because of toxicity; splenic toxicity is often associated with decreased incidences of leukemia in mice (296).

Furthermore, there can be no doubt that life-time rodent bioassays are not a completely reliable *test system* (297) and their interpretation is at times controversial (298). They are difficult to validate and are sometimes questioned with regard to their usefulness (299,300). Nevertheless, life-time rodent bioassays are widely accepted (301). Low sensitivity to detect a carcinogenic effect is of greater concern than low specificity (i.e., carcinogenic findings of no relevance to man (302,303)).

7.7. Safety Factors

If toxicity or an epigenetic carcinogenic effect of possible relevance to man is found, the single most important parameter to evaluate is the no-observed-adverse-effect-level (NOAEL) for toxic or carcinogenic effects in relation to the exposure. Is there a sufficient safety factor defined as exposure at the NOAEL in the most sensitive species (as far as this species is of relevance to man) relative to the maximal human exposure (dose/concentration and duration of exposure) occurring or intended? For the reasons given below, no strict rules are available. Generally, a safety factor of 10 for extrapolation from animal to man and an additional safety factor of 10 for inter-individual variation in man (for a total safety factor of 100) is considered acceptable. However, there are many compounds on the market with a considerably lower safety factor.

The *therapeutic indication* of drugs is particularly important. Lower safety factors are acceptable for life-saving indications compared to non life-saving indications. In general, higher safety factors are needed when children or young adults are the target treatment population, compared to medications used for patients towards the end of their natural life span. Are potentially safer alternatives available or has the substance in question a true advantage over the alternatives that would justify a minimal risk? How did regulatory authorities assess similar findings with other drugs? For chemicals other than drugs, generally none or limited human data are available. Furthermore, exposure to these agents happens accidentally or through contamination. Therefore, risk assessment tends to be more conservative for agrochemicals (304), other environmental chemicals (220,284), consumer products (305), residues in food (306), and chemicals at the workplace (307). The general principles are also summarized in textbooks of general toxicology or in various publications (e.g., Ref. 308).

Especially high safety margins are required for compounds that induce lesions which are considered irreversible. Lesions in the category include necrosis of neurons and ocular changes (particular retinal degeneration or degeneration of the lenses).

Certain authorities are also very cautious in case of toxicity to the reproductive organs, which could lead to sterility (besides the possibility of teratogenic effects).

8. REPORTING

8.1. Study Report

Good reporting of potentially adverse effects, including a candid discussion and scientifically sound conclusions are very important.

The *method section* must include a good description of the procedures followed during the study and for the evaluation. In particular, the

pathology methods must be described with reference to the standard nomenclature used for the histopathological diagnosis of lesions, the recording method for tumor multiplicity, and concomitant hyperplasia and neoplasia in the same organ or system, as well as the peer review procedures. The statistical evaluation procedures for tumors must be described, including pooling of groups and lesions. If additional investigations were performed on the material of the study in question, they have to be reported or a reference has to be given to indicate where the results are or will be reported. In general, additional mechanistic studies are reported separately.

The *result section* must comprise the following: an individual animal pathology report, with the findings for each animal in the study, namely macro- and microscopic findings including correlation with relevant in-life data, in particular the first occurrence of palpable masses (mainly rodent bioassays), and cause of death for animals that died or were killed in moribund state. In addition, summary pathology tables should be included, where treatment and control groups are identified separately, as are decedent and terminal sacrifice animals within each control/treatment group. Summary tables should be available at least for cause of death or moribund state/group, incidences/group for macroscopic lesions, nonneoplastic and neoplastic lesions as well as grading/severity for selected nonneoplastic lesions (as appropriate). For selected organs/tissue systems, one may want to consider including summary tables with combined benign and malignant tumors or hyperplasias as described in Sec. 5.6. Data regarding the normal variation of tumor incidences in the particular species and strain should be included as far as necessary.

In the *discussion section*, each finding should be addressed including decreased incidences of spontaneous lesions and their possible relationship with exposure or treatment (290,309) as well as pharmacokinetic and toxicokinetic considerations (135,229–231). The interpretation of non-rodent findings can be particularly challenging because of the relatively small number of animals used (310). A highly important aspect of the final discussion is that of possible mechanisms of toxicity (97,237,268,278,311,312).

8.2. Technical Documentation for Regulatory Authorities

In the discussion of an overall summary to be submitted to the authorities, a review of all internal (preclinical and clinical) and external (published) data of relevance to the issue should be performed and copies of all cited reports and publications submitted together with a final conclusion and recommendation to the authorities. Among other issues, the following items should be addressed: factors, which may have contributed to the findings; species-related particularities; pharmacology of the substance in relation to the toxicity/carcinogenicity findings; drug metabolism aspects; presence or absence of precursor lesions, and any other findings of importance. Particularly for

the risk assessment part, relevant human data must be included and discussed, if available. In this context, it is particularly important to point out the differences between man and test animals. One should also not be afraid to discuss—if appropriate—the worst case scenario; that is, risk assessment in the unlikely event that the toxicity or the proliferative effect seen in the test species is relevant for man. A conclusion is not just an opinion, but must be justified so one can attempt extrapolation from experimental animals to humans (311).

9. CONCLUSIONS

This overview is intended to introduce the reader to the science of pathology investigations and illustrate the value of pathology in preclinical development. Pathology provides mechanistic insight into pharmacologic and toxic effects. It also permits the preclinical development scientist to predict potential human outcomes to drug exposure by careful extrapolation from nonhuman studies.

Early microscopic assessment of nonhuman tissues may reveal subtle toxicity and subsequently lead to a timely discontinuation of the development project. Pathology assessments can also reveal potentially serious toxicities that might not become apparent e.g., until long-term clinical trials have been conducted.

In all cases, appropriate pathology support will increase the likelihood of safe drugs proceeding into and through clinical development. It is incumbent upon the preclinical development scientist to utilize pathology expertise early and frequently during the development of a drug candidate.

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One of the authors of this book chapter (RAE) managed this collection at its early stage. With the dissolution of the Institute of Toxicology, the collection passed into the custody of the Preclinical Safety Department of Novartis Pharma AG in Switzerland. Teaching slide sets with 24–30 duplicates of representative selected cases can be obtained for up to 4 weeks

at no costs with exception of those for mailing and insurance at casecollection@eurotoxpath.org

Microphotographs of representative lesions from this collection are part of a CD-ROM, edited by a group of pathologists coordinated by PG Germann. This CD-ROM is available from the European Society of Toxicologic Pathology (ESTP) (393).

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Principles of Toxicogenomics: Implications for Preclinical Drug Development

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1. INTRODUCTION

Modulation of gene expression is of paramount importance in the mechanisms of drug-induced toxicity, and major efforts are being made to determine the relevance of gene expression in response to drug exposure. DNA arrays offer the unique opportunity to explore the simultaneous gene expression of literally thousands of genes, up to entire genomes. Based on global gene expression profiles, and in conjunction with other molecular endpoints, reliable predictions of drug safety may now become feasible at early stages in drug development.

2. TRANSCRIPT PROFILING

Because of the completion of the human genome sequencing program, an assignment of all human genes to their chromosomal locations has been achieved. An important finding was an identification of a significant number of nucleotide polymorphisms, although not all nucleotide polymorphisms are of functional importance. At present, the difficulty lies in the dissection of functional and silent nucleotide polymorphisms that can be linked to a

given phenotype. Nonetheless, this information will greatly improve our understanding of individual responses during disease and drug treatment, and enable new concepts in personalized drug therapy. The translation of DNA information to gene expression is the second source of molecular analysis and permits a correlation between the genomic information and tissue-specific gene expression at any given time point and in response to a wide range of conditions, including pathological processes and physical traumata. Thus, the states of a cell, cell population, and the whole organism are determined by the genetic makeup as well as by the epigenetic and environmental influences, leading to tissue-specific gene expression.

Until very recently, it has been literally impossible to simultaneously assess the gene expression of hundreds of genes, because of the complex and cumbersome methodologies in molecular biology. Thus, gene expression profiling in diseased tissue could not be performed. Much of our knowledge in molecular medicine is therefore based on single gene expression analysis. Though the one-gene-one-disease paradigm has helped explain a set of diseases, it soon became obvious that the great majority of pathogenic processes have a multigenic basis and that the outbreak of a disease also depends on epigenetic influences. Therefore, large-scale or even genome-wide gene expression profiles are needed for a better understanding of the molecular events leading to metabolic deregulation and toxicity [Fig. 1](#), [Fig. 3](#) and [Fig. 4](#).

Since Southern introduced the blotting technique (1), the hybridization process has been used in a wide range of subsequently developed methods for the recognition and quantification of DNAs. In the beginning, a limited number of electrophoretically separated heterogeneous samples were immobilized on a membrane and tested with a single labeled cDNA probe—the classic northern blot—focusing on the expression of a distinct gene in different samples. Then, dot blots were used to enhance the number of addressable samples which were no longer separated. But only the reversal of this procedure—the arraying of multiple homogenous cDNAs and testing with a single heterogeneous labeled sample—made it possible to study the gene expression profile of thousands of genes in a given biological sample. With this procedure, a complex network of genes acting in concert can now be characterized, which permits a fundamental understanding of biological processes and fosters new concepts in molecular medicine and molecular toxicology [Fig. 2](#).

The basic technology behind DNA arrays and its application to drug discovery and drug development processes are described below.

3. METHOLOGICAL CONSIDERATIONS

3.1. Sample Generation, Labeling and Hybridization

Essentially, the handling of microarrays starts with the isolation of RNA from any given tissue or cells. The sample is then labeled either

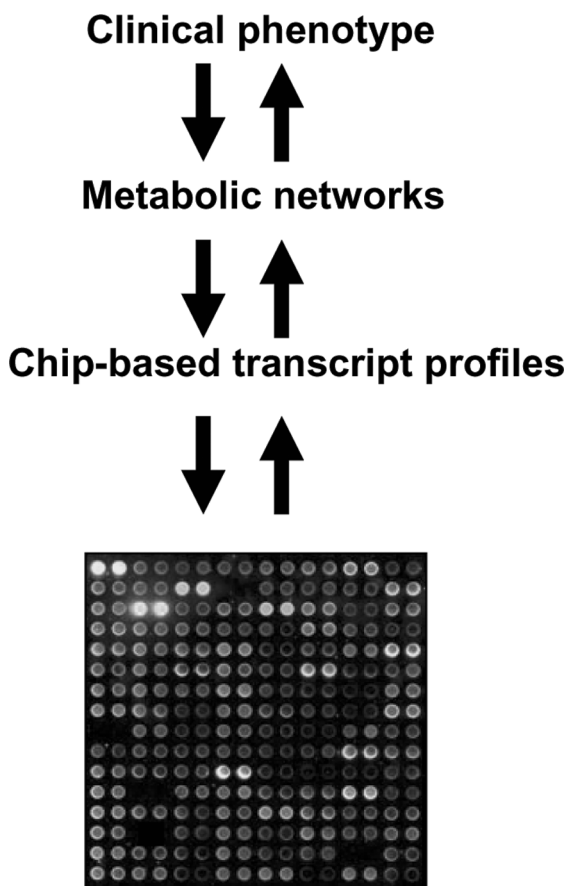


Figure 1 Differential gene expression in cultured human hepatocytes treated with an experimental drug. Each dot represents a gene. Green colour depicts repression where as yellow or red implies induction of mRNA transcripts. (See color insert [p. 7.](#))

enzymatically by converting the mRNA to cDNAs in an RT-reaction while incorporating labeled nucleotides or chemically by linking reactive molecules to the RNA. End-labeled primers may also be used for cDNA synthesis. The labeled molecules are then hybridized to the microarray, which is usually done overnight. If the incorporated label carries a fluorescent or radioactive tag, a direct or one-step labeling is possible and the hybridized array is ready for read-out. In case of an indirect labeling, the incorporated label needs to be biotinylated, haptenylated or carries alkaline phosphatase or horseradish peroxidase. This marker has to be contacted by a second

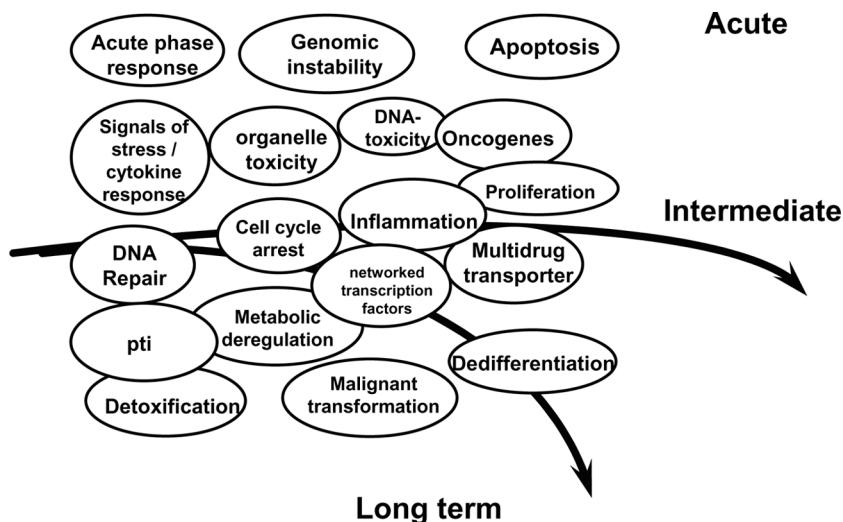


Figure 2 Gene networks linked to biological programs.

(and maybe third) molecule to produce a signal, which may be developed within 1 hr or up to 12 hr, depending on the method used, and which leads to an up to 20-fold signal amplification in a more or less linear fashion. The method of labeling determines on the one hand the sensitivity and hence the minimum amount of starting material for a successful hybridization and, on the other hand, the dynamic range and linearity of the relationship between sample amount and signal intensity. The most popular labeling procedure is the direct incorporation of fluorescent or radioactive-labeled nucleotides, as this method is fast and best understood in terms of robustness and reliability. When using direct fluorescent labeling, the amount of starting material should be at least 20–100 μg of total RNA, which corresponds to $1 \times 10^6 - 1 \times 10^7$ cells or 10–100 mg tissue, depending on the source of the sample. Sometimes, the amount of starting material is the limiting factor (e.g., when working with biopsies or microdissection materials or sorted cells) and, therefore, various amplification protocols have been developed for signal amplification based on an indirect labeling procedure. Another method of reducing the minimum amount of starting material by more than $1/10 - 1/1 \times 10^6$ is to linearly amplify the mRNA up to three times prior to the labeling. The amplification is done by a T7 polymerase-based in vitro transcription of double-stranded cDNA, which has been flagged with a T7 promoter sequence during the reverse transcription (2).

Currently, Cy3 and Cy5 are the most common dyes. However, strong efforts are being made to find better and cheaper fluorophors such as the Alex Fluor series.

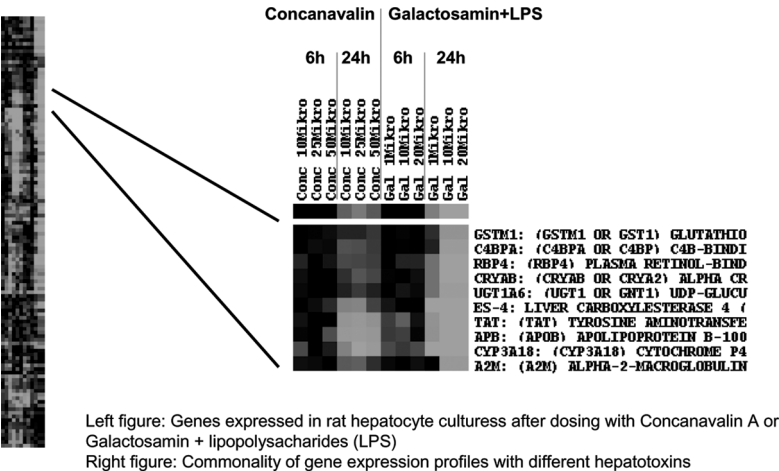


Figure 3 Hierarchical gene cluster analysis. Left figure: Genes expressed in rat hepatocyte cultures after dosing with Concanavalin A or Galactosamine + lipopolysaccharides (LPS). Right figure: Commonality of gene expression profiles with different hepatotoxins. (See color insert [p. 7.](#))

3.2. Image Capture and Analysis

Ideally, the control and study groups are assessed simultaneously; but for radioactive labeling, an analysis of only one group or condition per array is feasible. Hence, if two expression patterns have to be compared (e.g., treated vs. control), two distinct and separate arrays have to be hybridized. In addition, a varying background due to uneven labeling or washing, bubbles, nicks, scratches etc. impairs a precise quantification. Thus, results obtained with radioactive labels are better understood as “trends” rather than precise measurements (e.g., strongly increased/repressed, mildly increased, no change). Once again, no total quantitative measurement can be obtained. In contrast, by using various fluorescent labels (Cy3/Cy5), at least two groups can be simultaneously studied and directly compared with each other on a single array. The signals of both fluorophors are captured and digitally overlaid. This procedure, also known as two-color rationing, overcomes the bias between individual hybridization experiments if two arrays are to be compared. It is used with most solid surface arrays, with the exception of Affymetrix’s (Santa Clara, CA) GeneChips that are hybridized with only one labeled and amplified aRNA. In the Affymetrix approach, an internal normalization of the signals based on a number of controls is used to generate “absolute” expression intensities prior to comparing two expression patterns and determining expression ratios.

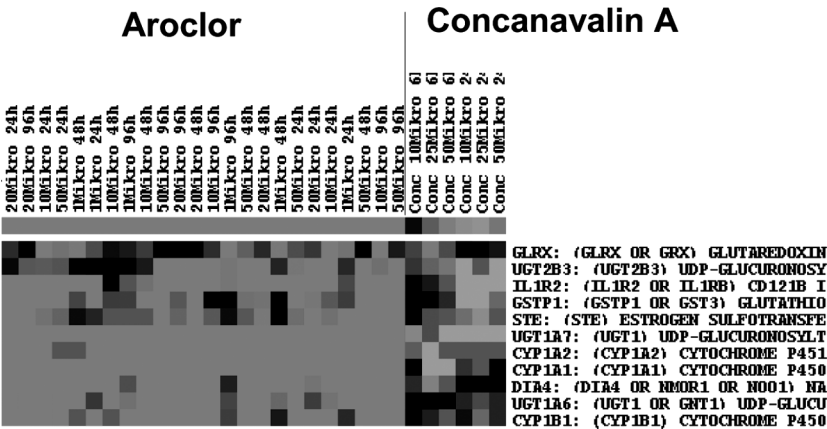


Figure 4 Differentiated gene expression of cultured rat hepatocytes treated with the hepatotoxins Aroclor 1254 a polychlorinated biphenyl or Concanavalin A. (*See color insert p. 8.*)

The signals on the arrays are captured either by a conventional phosphor imager or by a fluorescence imaging device. The latter may be divided into laser (confocal) scanner and charge-coupled device (CCD) camera-based systems. With respect to sensitivity, both systems are similar (<1 fluor molecule/ μm^2) in detecting a small number of transcript copies. Most of the CCD-based systems illuminate the whole array with a white light source, and a single picture is taken. Although this is a fast process, one needs to compromise between the captured area and the resolution, which is often not better than $30\mu\text{m}$. With cDNA spots having a diameter as small as $100\mu\text{m}$, only 9 pixels/spot are obtained, which is not a sufficiently accurate signal quantification. Moreover, the high depth of field may lead to halo effects and cause the capture of artifacts like dust at the bottom of the slide. Also, some kind of distortion at the edges of the picture has been observed which again influences the quantification procedure. Most of these problems are not observed with laser scanning devices (confocal or nonconfocal) (Axon Instruments, Inc. CA; GSI Lumonics, MA; Virtek Vision International Inc.), but the scanning process takes longer compared to CCD-based systems, as every pixel is exited and captured sequentially rather than in parallel. Due to the low depth of field, less artifacts occur; however, a lot of information may be lost, especially if the slide is not absolutely planar and in a horizontal position. Furthermore, as laser excitation is used, every wavelength needs its own light source, which reduces the flexibility in the choice of fluophors. An interesting approach is the arrayWoRx station of Applied Precision, Inc. (Issaquah, WA).

3.3. Microarray Formats

From an applicational point of view, microarrays should be divided into (i) tools for expression analysis (cDNA-arrays and oligonucleotide arrays) and (ii) tools for sequence analysis (re-sequencing, polymorphism detection etc., oligonucleotide arrays only). From a technical point of view, DNA arrays can be subdivided corresponding to the way they have been produced and to the kind of DNA they are carrying. First of all, DNA molecules are either synthesized in situ on the surface (oligonucleotides) or presynthesized and then spotted (oligonucleotides, cDNAs, and others).

With the in situ approach pioneered by Affymetrix Inc. (3,4), high-density oligonucleotides are synthesized by a photolithographic technology as originally developed for semiconductor chips. Nucleotides protected by a photolabile group are applied one after another to a silicon wafer to build a growing oligonucleotide chain. Photolithographic masks are used to cover parts of the surface, while uncovered parts are illuminated, which leads to the uncoupling of the protective group. Though a careful monitoring to ensure the fidelity of the growing oligomer is guaranteed, a step yield of approximately 95% limits the length of oligomer that can be synthesized to about 25 nucleotides. Transcript expression is measured with a series of oligonucleotides spanning the known sequence and additional partner oligonucleotides with one-base mismatches to the target sequence. As the oligonucleotides are relatively short, a hybridization stringency driven single nucleotide mismatch discrimination is possible and consequently, the technology can be applied to re-sequencing in addition to expression profiling. While there is no need to maintain a large collection of cloned cDNA molecules, there is a limiting factor concerning the flexibility of array configuration. The cost-intensive design and fabrication of photolithography masks restrict the production of arrays to very popular and common applications, thus leading to a relatively slow turnaround time in the development of new chips. In addition, the use of short oligonucleotides reduces the sensitivity, which necessitates the amplification of the starting material (aRNA).

The second technique for producing microarrays is the deposition of prefabricated oligonucleotides or cDNAs. This approach, promoted by the Brown laboratory of Stanford University (5), will be discussed in more detail. This popular technique is well suited for the rapid design and production of new arrays with different sets of cDNA probes. A collection of cDNA clones, a spotting robot for the deposition of the cDNAs, and a surface chemistry for the attachment of the cDNAs on solid surfaces (e.g., glass slides) are required for the production of these microarrays. The aforementioned factors (cDNA library, spotter, and substrate) undoubtedly also exert the greatest influence on the quality of a microarray, as defined by its low background, high signal intensity, grid accuracy, low spot variation, low intra- and inter-array variance, and reproducibility.

3.4. Substrates

The first kind of substrates used was a nylon membrane. Today, these membranes are still used. Probes are applied with 96 or 384 pin spotter to a density of up to 1176 cDNAs/approximately 13×9 cm (Clontech Laboratories, Palo Alto, CA) or approximately 18,000 cDNAs / 22×22 cm (Genome System, Inc.; Research Genetics, Inc; Eurogentec, Belgium; Genosys Biotechnologies, Inc.). These miniaturized dot blots bear some advantages which made them very popular, especially in the beginning when everybody was familiar with and had the equipment for membrane hybridization and radioactive labeling. Nevertheless, the power of microarrays only comes true when a solid substrate as glass or silicon is used. Some of the advantages are that fluorescently labeled samples can be used and that more than one sample can be analyzed simultaneously by incorporating different fluorophors. The spotting density is no longer limited by the substrate but only by the accuracy of the spotter pushing the density limit by two order of magnitudes. Consequently, the amount of hybridization volume is reduced to 1/1000. The greatest advantage lies in the handling of the solid substrate, which is much more robust and convenient and allows a full automation of the spotting as well as handling and read-out of the arrays.

Glass and silicon are not suitable for a stable attachment of DNA. Thus, different kinds of surface coatings have been developed. The coating of the microscope slides is a critical step in the production of cDNA arrays. Its quality is characterized mainly by a uniform coating, the absence of self-fluorescence, high DNA binding capacity, and little background after hybridization. Most of the coatings which have been developed make use of the reactivity of the nucleobases and lead to an unspecific crosslinking of the DNA to the surface e.g., silyl (reactive aldehyde), silane (reactive amino groups), or polylysine (5). These interactions can reduce the conformational freedom of the cDNAs and hence their affinity to complementary molecules in solution.

3.5. cDNA Library

While the coating heavily defines the sensitivity and reproducibility of the arrays, the quality of the arrayed cDNAs defines the reliability, the selectivity and the inter-species comparability of the gained signals or signal ratios. As stated before, oligonucleotide arrays may be used for expression profiling, having the advantage of being very selective. Thus, closely related sequences can be distinguished. In the aforementioned Affymetrix approach, at least 20 different oligo pairs per transcript are needed to insure reliable measurements of a single gene expression. This high level of redundancy is needed for accurate experimentation and a unique feature of the in situ photolithographic approach. Furthermore, spotting of 40 different oligos per transcript would not make any sense if the same results were

obtained by spotting longer and thus more reliable cDNAs. Therefore, most of the currently produced arrays rely on publicly available cDNA libraries that have been assembled by the integrated molecular analysis of genomes and their expression (IMAGE) consortium and are obtained from a number of distributors (Resource Center of the German Human Genome Project, Berlin, Germany; UK Human Genome Mapping Project, Hixton, UK; American Type Culture Collection, USA; Genome Systems, USA; Research Genetics, USA). A further point of consideration is the use of questionable or potentially wrongly annotated cDNAs, which greatly impacts data interpretation, but this is no inherent drawback for the use of array technologies. The most obvious reasons for false data interpretation are (i) poor annotation, (ii) repetitive elements, (iii) hybrid clones (DNA fragments merged from two different cDNAs during PCR), (iv) conserved sequences among gene families and (v) splice variants. In the case of IMAGE clones, it is well known that there have been problems with T1-phage contamination of some of the storage plates and that the clones have an error rate in sequence matches of approximately 20%; i.e., in 20% of the cases, the sequence given in the IMAGE database will not match the sequence of the clone in the corresponding storage plate. Though differently synthesized and with a potentially higher selectivity, one should keep in mind that every kind of oligonucleotide array faces the same problems concerning annotation, hybrid clones, conserved sequences, and splice variants if the sequences are not tightly controlled before they are used for the design of oligonucleotides.

In order to circumvent the discussed problems, a suitable subsequence is selected that fulfills the following conditions: (i) the length of the subsequence is between 200 and 400 bp, (ii) the region can be flanked by suitable PCR-primers in respect to primer length, T_M , base composition etc., (iii) the region does not cross-hybridize to other RNAs, i.e., (a) the region does not contain repetitive elements (Alu, B1, MIRs, and Microsatellites) (b) the region should have minimal sequence similarity (<80%) to related sequences from the same species, (iv) the region is not contained in an alternative splicing scenario (as far as known) to ensure that it is present in all relevant mRNA molecules. If there are indications of alternative polyA-usage, the region is chosen upstream of the first used polyA signal and (v) if possible, the region should not be too far from the polyA to give better yields in the RT reaction.

If possible, the flanking regions used for PCR-primer are selected in such a way that they are sufficiently well conserved between human and mouse or rat to allow amplification of clonable fragments from multiple species. The implication of this procedure is that one gets for each selected gene the same region of the gene for two or more species and therefore, virtually identical arrays are produced for different species but with of course species-specific cDNAs. Most certainly, this is the way forward for rational

and evidence-based cross-species comparisons with typical applications in pharmacology and toxicology.

3.6. Selection of cDNAs

For the generation of suitable cDNAs, searches in databases and the application of bioinformatic tools are needed, and usually one starts with a list of characterized cDNAs. These cDNAs are assigned to gene families according to sequence or structural elements and/or motifs. Using motif search algorithms and partly proprietary motif data bases, related cDNAs of a given gene family are added. Each cDNA is checked thoroughly for a suitable fragment as outlined above. As has been stated before, the fragments have a homogeneous length of about 200–400 bp. This is long enough for stable hybridization conditions and short enough to fulfill various quality criteria and to allow a strand-specific attachment to a substrate. Furthermore, the homogenous length of the fragments facilitates the production and normalizes the hybridization as the hybridization kinetics of each probe is comparable.

3.7. Spotter

Different spotting techniques for the production of microarrays have evolved which can be divided into contact and noncontact printing. Both contact and noncontact printers enable a sufficient density of up to 10,000 spots/cm². Contact printers are characterized by the fact that in order to deposit the probe on the substrate, there has to be a direct contact between the delivering tool and the substrate (BioRobotics Ltd., UK; Genetic MicroSystems Inc.; Genomic Solutions; GENPAK Inc.; Labman Automation Ltd., UK; TeleChem International). Various kinds of pins are dipped into the probe reservoir which leads to the uptake of a DNA-containing liquid. An aliquot [100–500 pL] of this liquid is then delivered each time the pin touches the surface. When solid pins are used, only one aliquot of liquid can be delivered. Split pins have a small uptake channel fabricated into the steel which allows the uptake of greater volumes for multiple dispensing. Major drawbacks are mechanical variation and long-term alteration of the print heads leading to varying spot sizes. Noncontact printers (ink jetting) have an active rather than passive uptake and delivery of the liquid driven by a syringe or membrane pump coupled with (a) a micro-solenoid valve (Cartesian Technologies, Irvine, CA) delivering >20 nL drops or (b) a piezoelectric pressure pulse (Packard Instrument Company; Rosetta Inpharmatics; GeSiM, Germany) delivering >50 pL drops. Especially the piezoelectric technology is rather complicated when compared to the contact printing, but it offers more flexibility especially with regard to the quality control and reproducibility of the spots. First of all, the quality and delivery of the drops can be tested with a video camera using stroboscopic light of the same pulse frequency and adjusted by changing the

pulse width, frequency, and voltage. Once optimal settings are selected, the amount of liquid delivered is constant and thus every spot is identical. By varying the number of drops per spot, the spot diameter and/or amount of material can be altered. Any kind of nucleic acids (oligonucleotides, genomic DNA, cDNA, and RNA) and in principle, almost any kind of solution may be spotted with this technique. Therefore, piezo-based sample delivery can be used for a wide range of applications, e.g., proteomics and combinatorial chemistry. Moreover, because of the active pumps, enough liquid for the production of thousands of arrays can be aspirated at once thereby reducing production time and interarray variances.

4. GENE EXPRESSION PROFILING—PRACTICAL CONSIDERATIONS

It has already been discussed that various types of microarray formats exist. Although it is not discussed in this review, we wish to point out that microarrays are also used for SNP detection and genotyping (6,7). When applying microarray technologies, the crucial question is: which microarray configuration fits best for a given research program? In the following, the utility of microarrays for: gene discovery, “genome-wide” expression patterns (transcriptome analysis), functional analysis of new genes, drug validation, and pharmaco- and toxicogenomics are discussed.

The importance of gene discovery lies in the identification of gene expression profiles and its correlation with the biological states of cells, tissues, and organs during disease or upon drug treatment. This enables the identification of new drug targets and a molecular medicine-based understanding of disease. For example, Alizadeh et al. (8) reported the identification of tumor-specific expression patterns in hematopoietic (lymphoid) cells. In gene discovery programs, it is essential to simultaneously study as many genes as possible, but this requires a profound understanding of the genome. In the foreseeable future, the genomes of common laboratory animals will be unraveled and reliable cross-species comparisons (human and animal) and predictions of biological responses will thus become possible.

In gene discovery programs, an approximate expression intensity of a given gene is sufficient, so that it does not make a great difference if the arrays are membrane- or glass-based. Currently, various arrays are available for the large-scale/genome-wide study of human, model organism, or bacterial genes. It should be noted that every cDNA which is on an off-the-shelf array has been cloned and presumably sequenced before. In consequence, this means that (i) the array hybridization is more precisely termed function or expression discovery than gene discovery and (ii) most of the genes are not low copy number genes. The only way to find truly new (and patentable) genes is to generate new libraries or to use one of the numerous alternative techniques, such as serial analysis of gene expression, SAGE (9), suppression

PCR (10), differential screening and subtractive hybridization (11), differential display (12), SEREX (13) or Proteomics (14). In the SAGE approach, short sequence tags unique to each transcript are generated and concatamerized. Upon sequence analysis, approximately 25 or more independent tags can be identified from each sequencing lane while the expression intensity of a gene directly corresponds to the number of distinct tags counted.

“Genome-wide” expression pattern discovery aims at elucidating gene interactions, pathways, and recognition of gene groups acting in concert (15). For this purpose, high-density off-the-shelf arrays or customized arrays are available. In this instance, precise quantification of gene expression is of interest and therefore, glass- or silicon-based arrays are better suited.

For functional analysis of new genes, a limited group of genes are of interest, and one would like to obtain more information in different biological scenarios by testing the expression intensity in numerous biological samples. The exact expression level is of interest, thus low to medium glass-based arrays are the right choice.

Expression arrays used for drug validation, diagnostics, pharmacogenomics, or toxicogenomics can be envisaged in a two-step scenario. In a first step, when little is known about a disease, a drug, or toxicological endpoints, expression analysis with high-density arrays or an alternative method as described above with appropriate controls should be performed (8,16–18). This analysis leads to the recognition of a subset of genes which can be correlated with a disease, metabolic deregulation and/or intoxication, or with the effects/side-effects of a class of drugs. Although attractive, the genome-wide transcriptome analysis has, at present, several drawbacks: First, the immense cost associated with studying genome-wide expression is the major shortcoming of this approach, especially when dose and time studies are being investigated in parallel. It is therefore, not surprising that genome-wide expression studies are not conducted with the usual replicates of experimentation though standards for quality have been defined (see MIAME for further details). Second, data mining and analysis are difficult and complicated because a lot of redundant data can be generated with only 5–10% of randomly selected genes showing differential expression. Third, high-density arrays rely on EST libraries, which are not of best quality, and it is strongly recommended to corroborate the results by additional techniques.

5. CASE STUDY: ELUCIDATION OF TERATOGENIC MECHANISM(S) OF TOXICITY OF A DEVELOPMENTAL DRUG, EMD 82571

The drug EMD 57033 has been characterized as a calcium sensitiser and was developed to treat coronary heart disease. Calcium ions play a pivotal role in the control of the myocardial contractility, and a rise in contraction force can be elicited by either increasing the transient free Ca^{++} concentration and/or

by augmenting the myofibrillar sensitivity to Ca^{++} . Traditionally, drugs have been developed which raise the force of contraction by strengthening the myocardial contractility ultimately by raising the Ca^{++} concentration. Classes of medications in this category include β -sympathomimetics, phosphodiesterase III (PDE_{III}) inhibitors and cardiac glycosides.

A pro-drug of EMD 57033, EMD 82571, was the first drug developed which was shown to increase contraction primarily via calcium sensitisation. It also has PDE_{III} inhibitory properties, contributing to its overall cardiovascular profile. The compound acts through “down-stream” mechanisms at the level of the actin–myosin cross-bridges by increasing the contribution of each cross-bridge to the contractile force (Merck KGaA internal report).

The acute toxicity of EMD 82571 in rodents was shown to be low. However, female rats were considerably more sensitive, and this is due to the large differences observed in the first pass metabolism. Bioavailability of the active drug was only 9% in male rats, but 60% in the female rat. Repeat dose studies showed a “no observable effect level” of 10 mg/kg in both rats and dogs. At higher doses, mild toxicity was observed, with the principal target organ being the liver, which increased in weight and demonstrated centrilobular hypertrophy associated with increases in liver enzymes. Histologically, perivascularitis and vacuolation of the centrilobular hepatocytes was observed. The Ames test ($\pm$ S9), the rat micronucleus test, and the mouse lymphoma cell assay (+S9) were all negative. Mutation frequencies were slightly increased in the absence of S9 activation in the mouse lymphoma cell assay.

Embryo-fetal toxicity was studied using EMD 82571, EMD 57033, and the ester group (*N*-methylpiperidinol) removed to form EMD 57033 in rats. Administration was oral and from gestation days 6 to 17. All high dose (150 mg/kg) fetuses were malformed after EMD 82571 treatment. The most common abnormalities were exencephaly, micrognathia, and palatoschisis (Fig. 5). Treatment with EMD 57033 or *N*-methylpiperidinol did not cause an increase in the frequency of malformations, even up to concentrations of 300 mg/kg. (Merck KGaA internal report) Fig. 5.

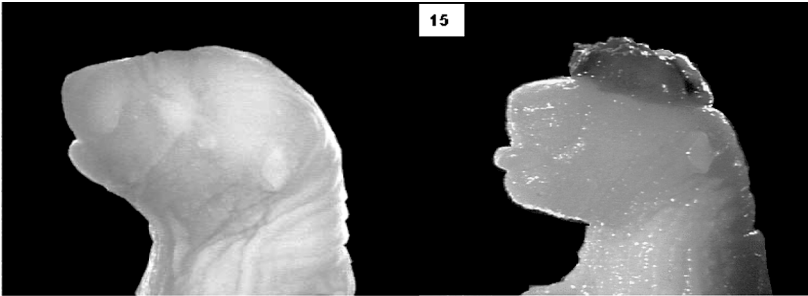
5.1. Hypothesis Driven Gene Expression Array

5.1.1. Gene Selection

Two hundred and forty-six Genes were selected based on specific genes reported to be involved in teratogenic processes and fetal development. Other additional genes were also selected based on their putative role in EMD82571's mode of action and its toxicological profile (see Table 1).

5.2. Study Design: In Vivo Embryotoxicity Study

Pregnant female Wistar rats were dosed daily with either EMD 82571 (50 mg/kg or 150 mg/kg) or retinoic acid (RA, 12 mg/kg or 20 mg/kg) on



Control

Treated

Figure 5 Depicted is the exencephaly in fetal rats as a result of treatment with EMD 82571. (See color insert [p. 8.](#))

gestational days 6–17 ([Table 2](#)). Retinoic acid was chosen as a positive control due to its very well-documented effects on developing fetuses—including similar malformations observed for EMD 82571 (19–21).

On gestational days 12 and 20, the female was sacrificed and the fetuses removed (visible malformations were noted; [Table 3](#)). The maternal

Table 1 Rationale for Gene Selection of Focused cDNA Array

Developmental regulators	BMP's, WNT's, HNF's, Hox's, PAX's, RXR's, RAS's, CEBP, CREB, etc
Extracellular matrix/cytoskelton structural proteins, matrix metalloproteinases, adhesion molecules	Collagens, laminins, integrins, fibrillins, micrifibrillar proteins, bone specific proteins, calcium handling proteins, MMP's, TIMP's, endonucleases, cartilage, osteopoetin, microfibrillar proteins, aggrecan core proteins
Cell cycle	DNA polymerase, topoisomerase, E2F1, CDK's, replication factors, ribosomal proteins, GADD's, etc
Growth factors/nuclear factors	Connective tissue growth factors, granulocyte colony stimulating factor, TGF, TGFR's, IGF, IGFR, PDGF's, VEGF's, CAR, CXR, PXR, PPAR, etc
Acute phase response/heat shock response	Heat shock proteins, ubiquitin, α 1-AT, CRP, α 2-macroglobulin, etc
Detoxification	CYP'S, UGT's, ST's, steroid metabolism, FMO's, HPRT, etc
Metabolism	Protein, lipid, carbohydrate

Table 2 In vivo Developmental Study Details—Treatment

Treatment	Days of treatment	Day of section	Number of animals
Control	d 6–11	d 12	5
50 mg/kg EMD 82571	d 6–11	d 12	5
150 mg/kg EMD 82571	d 6–11	d 12	5
Control	d 6–11	d 20	5
50 mg/kg EMD 82571	d 6–11	d 20	5
150 mg/kg EMD 82571	d 6–11	d 20	5
Retinoic acid ^a	d 6–17	d 20	5

^aDose chosen according to Emmanouil-Nikoloussi et al., 2000.

liver, whole embryo (day 12), fetal liver (day 20) or fetal bone (cranium and mandibular bone) were removed and immediately snap frozen in liquid nitrogen from all animals in the study.

5.3. Gene Expression Experimental Design

5.3.1. cDNA-array Production

Defined 200–400 bp fragments of selected 246 cDNAs derived from rat liver tissue were cloned, amplified, purified, and checked for size and purity, on an agarose gel. Amplified inserts were gridded on treated glass slides (Memorec, Germany) using an ink jet micro arrayer (Luigs & Neumann, Ratingen and Memorec, Germany).

5.3.2. Sample Labelling and Hybridization

Total RNA was extracted and 40 µg of total RNA was used for each hybridisation. Control samples were labelled with Cy3, treated samples with Cy5. Hybridisation was carried according to standard procedures.

5.3.3. Data Analysis and Normalization

Arrays were read using ScanArray3000 (GSI Lumonics). Signal intensities of Cy3 and Cy5 samples were normalized to the median of the housekeeping genes and were determined using standard procedures used in the laboratory.

5.4. Results

Two hundred and forty six genes were selected based on their putative role in EMD82571's mode of action and their importance in known teratogenic pathways and analysed using a custom made "Bio-Chip."

There was clear induction and suppression of many genes after treatment with EMD 82571 and RA in both maternal liver and the different embryonic tissues taken. These could be grouped into compound specific gene changes and common gene changes (Table 3).

Table 3 Malformations Caused by High Dose EMD 82571

Female no.	Individual external observation (No.of fetuses)					
	Number of fetuses	Exencephaly	Exencephaly, Micrognathia/agnathia	Exencephaly, facial cleft	Exencephaly, Micrognathia/agnathia, facial cleft	Within normal limits
1	6	3	1	1	—	1
2	11	—	6	—	5	—
3	10	—	—	—	—	10
4	10	—	2	—	1	7
5	13	6	—	4	—	3

Specific changes caused by EMD 82571 included: (i) induction of: HSC 73 (heat shock cognate 73 Kd protein), Fen 1 (Flap endonuclease 1), HSP105, Collagen 2A1, Bone morphogenic protein 7 (Bmp 7), connective tissue growth factor precursor (CTGF) and (ii) suppression of: epithelial-cadherin precursor (Cdh-1), alpha-1-antitrypsin precursor (AT1).

Genes which were commonly induced by both EMD 82571 and RA included: hypoxanthine-guanine phosphoribosyltransferase (HPRT), GAPDH, glucuronosyltransferase 2B2 (UGT2B2), cytochrome P450 2E1, cytochrome P450 7A1, and insulin like growth factor II (IGFII).

It is clear that both EMD 82571 and RA caused significant changes in gene expression in both the maternal liver as well as the target tissue. These changes can be specifically linked to general cellular stress (e.g., Hsc 73, Hsc 105, DNA topoisomerase IIb, and Fen1), but more importantly to specific changes during fetal development—Bmp7, Collagen 2A1, and Ctgf. The induction of HPRT could explain the significant ($P < 0.05$) reduction in serum urea levels observed during previous *in vivo* toxicity studies. It is also known that HPRT oxidase inhibitors also cause an increase in HPRT activity, leading to teratogenic effects, similar to these seen in these studies.

It is hypothesised that the metabolic modulation observed could lead to changes in bile acid synthesis. This would lead to high levels of circulating bile acids (such as, deoxycholic acid), which have been reported to cause fetal resorptions, as well as significant increases in fetal malformations (22). These effects have also been reported in humans. The Zellweger syndrome (or cerebro-hepato-renal syndrome) is a congenital disorder characterized by cerebral dysfunction, craniofacial dysmorphic features, transient cholestasis, and renal cysts. Patients fail to thrive, and usually die in their first year of life. Several biochemical abnormalities have been observed including elevated levels of coprostanic acids and the C-29 dicarboxylic bile acid. Therefore, it was postulated that EMD 82571 acts directly on the maternal liver and subsequently causing the teratogenic effects observed.

5.4.1. Number of Genes Affected (by class) After Treatment with either Retinoic Acid (RA) or EMD 82571 in Embryo Liver and Bone Tissue

Many congenital malformations are produced during gastrulation and neurulation stages of embryogenesis. Altered maternal metabolism in rats has been reported to have a direct impact on the embryo, or even an indirect effect via disruption of the nutritive function of the yolk sac. It is essential to remember that the metabolic status of the embryo is rapidly changing and that the embryo will respond differently at different time of gestation. If the mother is compromised in any way, this will have a knock-on effect on the fetuses.

Interestingly, both EMD 82571 and RA induced the expression of GAPDH, a commonly used “house keeping” gene. This suggests that such

simple normalization procedures will not be adequate for large gene expression studies.

An attempt was made to discern from literature the genes that are the most relevant for the application. This approach is a completely closed system that will only provide results on strictly predetermined genes, which may already be well characterized. This microarray will not be representative of the entire genome. The advantage of smaller size and reduced complexity is that it facilitates the task of making a high specificity, high quality microarray for quantitative use. This is useful for focusing on mechanisms of action, when they are known. Other advantages of using such a hypothesis-driven microarray include costs, manpower, ease of use, and ease of data interpretation. However, the major disadvantage is the limited number of genes chosen. No amount of forethought can really predict all the important gene expression changes that may occur. In addition, the rat genome has yet to be completely sequenced and, as a consequence, annotation is difficult. At the moment, one of the most advanced rat array systems available is that from Affymetrix (A chip has 15,000 annotated genes and the B chip a further 13,000 ESTs).

6. TURNING DATA INTO KNOWLEDGE

It is well established that the expression of certain genes in intoxicated or diseased tissue is altered (over-expressed or suppressed). Continued elucidation of the genetic changes that drive toxicity and/or cancer progression is yielding new and mechanistically based information (Fig. 6). Nucleic acid based expression patterns are proven valuable tools for an early detection of toxicity and disease, for the confirmation or exclusion of a cancer diagnosis based on suspicious and nondiagnostic material, and for an assessment of the tumor burden and of the response to preventive approaches. Toxicity is a heterogeneous event driven by a complex array of genetic changes that may lead to an uncontrolled growth and potentially metastatic spread. The changes in gene expression profiles can be used as molecular fingerprints and for the early detection of metabolic deregulation. Based on our emerging knowledge of the gene expression profiles, various databases to provide novel information on drug profiling studies and to predict organ- and tissue-specific toxicity are being developed. Hence, the determination of patterns of altered gene expression will provide a rational basis for drug development decisions in a cost-effective and timely fashion. In general, toxicogenomic research will provide fundamental information on the mechanism of action of chemicals at the transcriptional level; and modulation of gene expression may predict toxicities not otherwise observed in preclinical or clinical drug studies.

In conclusion, microarrays are the tools for molecular and evidence-based medicine and hold promise to greatly impact the drug discovery

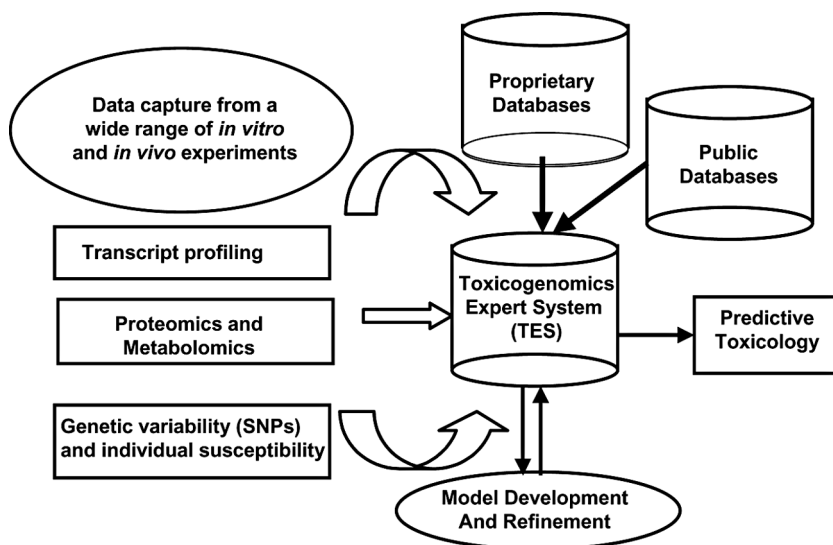


Figure 6 Data flow and data capture in a toxicogenomic expert system.

and drug development in a cost- and time-efficient manner. In conjunction with reference databases, more informed decision-making could occur at early stages in the drug development process rather than after more costly animal or human trials Fig. 6.

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Utilizing the Preclinical Database to Support Clinical Drug Development

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The primary goal of the preclinical phase in drug development is to generate information that can be used for the rational and informative conduct of clinical studies. This goal of the preclinical phase can be achieved through use of a variety of in vitro or ex vivo technologies such as isolated organs and tissues, cell cultures, cellular fragments, subcellular organelles, receptors, ion channels, transporters and enzymes, and whole animal in vivo investigations (1–3). Traditionally, many preclinical studies support early clinical drug development, i.e., clinical pharmacology, pharmacokinetic (PK), and pharmacodynamic (PD) studies; however, various preclinical studies are conducted to support late clinical development as well as post-marketing needs (4,5).

From the perspective of clinical drug development, the aims of preclinical studies should be evaluations of both safety and efficacy in various experimental settings of animal species that can be integrated into the information database for the safe and effective use of the drug in humans. More specifically, the preclinical safety evaluation should focus on identification of (1) an initial safe dose and subsequent dose escalation schemes in humans, (2) potential target organs for toxicity and their reversibility, (3) safety parameters for clinical monitoring, and (4) at-risk populations (6,7). Likewise, the specific aim of efficacy evaluation during the preclinical

phase is primarily to elucidate the PD characteristics of the agent and their impact upon the therapeutic activity (8). Therefore, the determination of the PK profile of the drug and/or the metabolites in animals as a guide to these characteristics in humans is critical. Additionally, the development and use of biomarkers in animal models are strongly recommended because these can demonstrate early signals of efficacy and toxicity in humans (9–14).

In this chapter, some real drug development cases will be introduced, in which the preclinical database was adequately utilized to provide drug developers with supportive, and sometimes critical, evidence necessary to design efficient and informative clinical development programs, thereby facilitating final regulatory approvals. These examples will be followed by a brief discussion on the implication of using the preclinical database in a specific drug development setting.

1. UTILIZING THE PRECLINICAL DATABASE TO SELECT HUMAN DOSE

The selection of dose for the first-time-in-man (FTIM) study is one of the most important and difficult decisions to be made when entering the clinical phase of drug development. The dose for the FTIM study has to be small enough to not to cause harm to the subjects, but, at the same time, a starting dose that is too small must be avoided since it will increase the time needed to reach the maximum tolerated dose (MTD) or the clinically efficacious dose. Many methods to determine the safe human dose have been introduced with mixed results (15–17).

1.1. Determination of Starting Dose: a Regulatory Agency's View

The US Food and Drug Administration (FDA) released the Draft Guidance for Industry and Reviewers for Estimating the Safe Starting Dose in Clinical Trials for Therapeutics in Adult Healthy Volunteers (“Draft Dose Guidance” hereafter) in December, 2002, outlining a standardized process for deriving the maximum recommended starting dose (MRSD) for adult healthy volunteers for first in human clinical trials of new molecular entities (18). The Draft Dose Guidance defined the MRSD as the highest initial dose in a clinical trial that is predicted to cause no adverse reactions in adult healthy volunteers.

The process for selecting the MRSD is summarized in [Fig. 1](#). The MRSD is selected after the determination of the no observed adverse effect levels (NOAELs), based upon the analysis of toxicity data from preclinical animal studies. Although only the NOAEL is used in the first step of the algorithm, other data (exposure/toxicity relationships, pharmacologic data, or prior clinical experience with related drugs) will affect the choice of the most appropriate species, scaling, and safety factors in later steps.

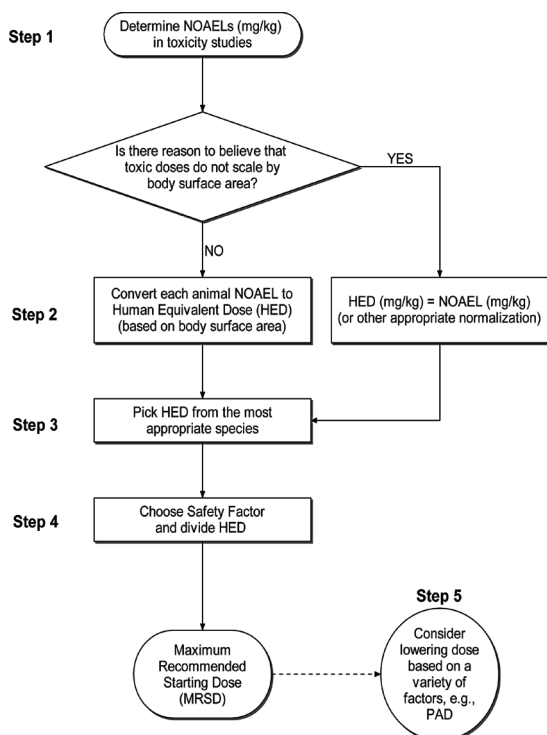


Figure 1 Selection of the maximum recommended safe starting dose for drugs administered systemically to normal volunteers. PAD: pharmacologically active dose. (From Ref. 18.)

The NOAEL for each species tested should be converted to the human equivalent dose (HED) using appropriate scaling factors. The Draft Guidance strongly recommends that the conversion be based on the normalization of doses to body surface area for most systemically administered therapeutics. In some cases, however, extrapolating doses based on other parameters (e.g., body weight) may be more appropriate; therefore, this decision should be based on the data available for the individual case. The body surface area normalization and the extrapolation of the animal dose to human dose should be done in one step; the NOAEL for each species is divided by the appropriate body surface area conversion factor, a unitless number that converts mg/kg dose for each animal species to the mg/kg dose in humans. This results in a HED, which is equivalent to the animal's NOAEL on a mg/m² basis. The species that generates the lowest HED is called the most sensitive species, but may not necessarily be the most appropriate species.

When information indicates that a particular species is most relevant for assessing human risk, the HED for that species should be used in subsequent calculations, regardless of whether this species was the most sensitive. This case is common for biologic therapies, many of which have high selectivity for binding to human target proteins, and limited reactivity in species commonly used for toxicity testing. In such cases, *in vitro* binding and activity studies should be done to select appropriate, relevant species before toxicity studies are designed (7). Additionally, a species might be considered an inappropriate toxicity model for a given drug if a dose-limiting toxicity in that species was concluded to be of limited value for human risk assessment (based on historical comparisons of toxicities in species to those in humans across a therapeutic class). In this case, data from that species should not be used to derive the HED. Without any additional information to guide the choice of the most appropriate species for assessing human risk, the most sensitive species is used, because using the lowest HED would generate the most conservative starting dose.

A safety factor should then be applied to the HED to increase assurance that the first dose in humans will not cause adverse effects. The use of the safety factor is based on the possibility that humans may be more sensitive to the toxic effects of a therapeutic agent than predicted by the animal models, that bioavailability may vary across species, and that the models tested do not evaluate all possible human toxicities. In general, a safety factor of 10 is recommended, and the MRSD should be obtained by dividing the HED by the safety factor. Safety concerns or design shortcomings noted in animal studies may increase the safety factor, and reduce the MRSD further. Alternatively, if the pharmacologic class is well characterized, with extensive human clinical and preclinical experience, information about the class may allow reduction of the default safety factor and an increase in the MRSD. The process described here derives the MRSD; a dose lower than the MRSD can be used as the actual starting dose. The MRSD is in units of mg/kg, a common method of dosing used in phase 1 trials. As previously stated, for purposes of initial clinical trials in adult healthy volunteers, the HED should ordinarily be calculated from the animal NOAEL. If the HED is based on an alternative index of effect, such as the pharmacologically active dose (PAD), this exception should be clearly stated in descriptions of starting dose calculations in the study protocol.

The guidance document described here is under critical review within the FDA and throughout industry. While many of the concepts presented within this draft guidance should be considered when determining the first dose in man, it can be expected that this guidance will undergo revision. As always, sound and rigorous scientific principles must form the foundation for any initial human dose rather than strict adherence to a general guidance algorithm.

1.2. Mechanistic Approaches to Determine Human Dose

Although it is intended to offer a pragmatic and conventional standard method as for establishing the MRSD, the FDA's Draft Dose Guidance, in the current form, is incomplete; it does not mention or call sufficient attention to other scientifically valid and more mechanistic approaches (19). For example, the Draft Dose Guidance placed little emphasis on the animal pharmacokinetic–pharmacodynamic (PK–PD) modeling approach, stating that, in the majority of new investigational new drug applications, animal data are not available in sufficient detail to construct a scientifically valid PK model whose aim is to accurately predict an MRSD (18). This view, however, appears inconsistent with other recent FDA guidelines that emphasize the importance of PK–PD relationships in establishing the safety as well as the efficacy of a drug in development (20,21). In other words, the Draft Dose Guidance has focused only on dose, which does not necessarily guarantee a specific exposure. Therefore, mechanistic approaches that take full advantage of existing preclinical information are needed to estimate the dose for a FTIM study.

Recent developments in advanced PK–PD modeling and simulation technology can be applied to design better informed clinical studies using existing data (22–26); this topic is discussed in detail in [Chapter 6](#). In some cases, PK–PD modeling and simulation can be also used to bridge the gap between preclinical and clinical phases of drug development. For example, PK–PD modeling and simulation were actively used to design the FTIM and the proof-of-concept studies of a new neurological agent using only preclinical data without the support of any human *in vivo* information; rhesus monkey and human PK were initially estimated using allometric scaling on data collected in dogs, cynomolgus monkeys, and rats (15). A PK–PD model was then derived from a study conducted in rodents and validated by comparing the model predicted response to that observed in a positron emission tomography experiment conducted in rhesus monkeys.

1.3. Preclinical Database to Determine Human Effective Dose

In some cases, preclinical data are the sole source of information by which the human effective dose (i.e., the dose for proof-of-concept study) has to be determined. For example, Palivizumab (Synagis[®]), a humanized monoclonal antibody against the Respiratory Syncytial Virus (RSV), was approved by the US FDA in 1998, and the indication was prophylaxis of serious lower respiratory tract disease caused by RSV in pediatric patients at high risk of RSV disease (27). Three *in vitro* neutralization studies (i.e., microneutralization assay of RSV, plaque reduction neutralization assay, and neutralization of clinical isolates) were conducted during the preclinical phase, in which palivizumab was shown to inhibit RSV replication (28). Additionally, *in vivo* intravenous (IV) treatment, intramuscular (IM) prophylaxis, and IV

prophylaxis studies were conducted using cotton rat animal models loaded with RSV, where the rats were challenged intranasally with 10^5 plaque forming unit (pfu) of RSV, and lung tissue was collected and pulmonary viral titers were determined. These studies showed that palivizumab was effective in both prophylaxis and treatment of the RSV pulmonary infection, and serum concentrations of $\geq 40 \mu\text{g/mL}$ were shown to reduce the pulmonary RSV replication in the RSV cotton rat models. For example, in the IV prophylaxis study, a reduction in RSV titer ≥ 100 -folds (i.e., 2 logs) corresponded to a mean serum antibody concentration of 25–40 $\mu\text{g/mL}$ at the time of challenge (Table 1).

The human PK studies were conducted in infants and children during the RSV season, and the mean trough concentration after single IM and IV doses of palivizumab (15 mg/kg) were 49 and 60.6 $\mu\text{g/mL}$, respectively. In addition, after 4 monthly IV doses of palivizumab 15 mg/kg, the mean trough concentration was 96.9 $\mu\text{g/mL}$ (29). Consequently, 15 mg/kg was selected as the human effective dose for the phase II and III studies with palivizumab largely based on the preclinical RSV lung infection cotton rat model and the PK profiles obtained in the phase I studies.

It is interesting to note that the clinical reviewer of palivizumab found the sponsor's approach for dose selection acceptable even though no dose–response relationship was formally investigated in the target population, i.e., pediatric patients at high risk of RSV disease (29). The clinical reviewer commented that, since the cotton rat model is a fairly well accepted one and that, due to its low incidence, it would have been very difficult to conduct a dose–response study using RSV hospitalization rate as the primary

Table 1 Prophylaxis by Intravenous Pavilizumab Administration in Cotton Rats Innoculated with RSV

Treatment	No. animals	Dose (mg/kg)	Concentrations of human IgG ($\mu\text{g/mL}$)	Lung viral titer (pfu ^a /g)
BSA ^b	15	10	0	$1.3 \times 10^5 \pm 1.2$
Pavilizumab	7	0.312	2.67 ± 0.60	$4.6 \times 10^4 \pm 1.5$
Pavilizumab	17	0.625	5.27 ± 0.27	$2.7 \times 10^4 \pm 1.3$
Pavilizumab	18	1.25	10.1 ± 0.29	$3.3 \times 10^3 \pm 1.4$
Pavilizumab	17	2.5	28.6 ± 2.15	$9.6 \times 10^2 \pm 1.5$
Pavilizumab	15	5.0	55.6 ± 3.43	$1.3 \times 10^2 \pm 1.2$
Pavilizumab	18	10.0	117 ± 5.09	$1.0 \times 10^2 \pm 1.0$

^aPlaque forming unit.

^bBovine serum albumin (control).

Source: Ref. 28.

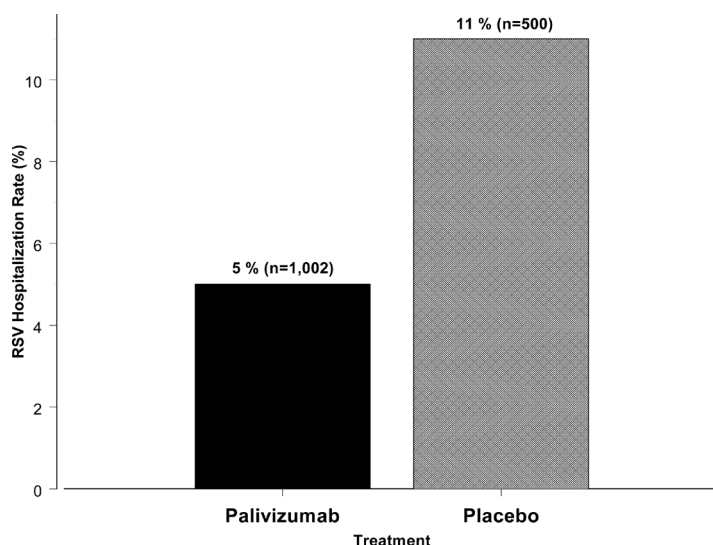


Figure 2 RSV hospitalization rates by palivizumab (15 mg/kg *IM*, monthly for 5 months) and placebo, $p < 0.0001$, 2-sided Fisher's exact test. (From Ref. 29.)

endpoint in human patients, the utilization of the preclinical information seemed reasonable.

Using 15 mg/kg as the human effective dose based on the preclinical database, a randomized, double-blind, placebo-controlled, multiple-dose (IM monthly injection for 5 months) phase III study of palivizumab was conducted in infants and children at high risk for severe RSV disease. The RSV hospitalization rate was used as the primary endpoint, and was statistically lower in the palivizumab treated group compared to that in the placebo-treated patients (Fig. 2), confirming that the dose selection approach was appropriate. This palivizumab case adequately exemplified how informative the preclinical database can be to select the human effective dose when the clinical dose-response studies are difficult or impossible to conduct.

2. UTILIZING THE PRECLINICAL DATABASE TO SUPPORT THE EFFICACY CLAIM FOR REGULATORY APPROVAL

Preclinical efficacy findings, if coupled with the well understood pathophysiology of a disease and the mechanism of action of a drug, could serve as the confirmatory evidence to support the conduct of a single phase III clinical trial for regulatory approval (30). This is a huge saving in terms of both time and resources for drug developers given that the current FDA position is to require at least two adequate and well-controlled phase III studies, each

convincing on its own, to establish effectiveness for final regulatory approval (31). Preventive vaccines are good examples; one adequate and well-controlled clinical trial may be supported by compelling animal challenge/protection models, if accompanied by human serological data, passive antibody data, or pathogenesis information demonstrating that there is a previously accepted correlation with clinical effectiveness and serological response data (20).

In some cases, efficacy data from animal models have been the most important or the sole confirmatory evidence supporting the final regulatory approval. For example, CelebrexTM (celecoxib) was approved by the FDA in 1999 for the reduction of the number of adenomatous colorectal polyps in patients with familial adenomatous polyposis (FAP), as an adjunct to usual care (e.g., endoscopic surveillance, surgery) (32). This was a supplemental new drug application (sNDA) for celecoxib, first approved in 1998 for the relief of the signs and symptoms of osteoarthritis and rheumatoid arthritis (33).

For the FAP indication, the sponsor submitted the results of a single randomized, double-blind, placebo-controlled, dose-response phase III study, in which a total of 83 patients received either placebo, celecoxib 100 mg b.i.d. (twice a day), or celecoxib 400 mg b.i.d., respectively, for 6 months. Patients on the celecoxib 400 mg b.i.d. showed a statistically significant reduction (mean = 28%) in the number of colorectal polyps from the baseline, which was used as the surrogate endpoint (34). A dose-response relationship was also observed (Table 2).

A significant body of evidence suggested that the cellular expression of cyclooxygenase-2 (COX-2) is prominent in some tumors, e.g., colon (35–37), skin (38), prostate (39,40), lung (41,42), and breast (43,44), as well as precancerous lesions including the adenomatous polyp (45,46). Based on this understanding of the role of COX-2 in cancer pathophysiology, celecoxib was evaluated in two animal models of colon cancer (34,47). In the adenomatous polyposis coli (APC) mutant Min mouse model, which represents a

Table 2 Reduction in Colorectal Polyp Counts by Treatments

Treatment	No. patients	% Reduction	<i>p</i> -value ^a
Placebo	15	4.5	
Celecoxib 100 mg b.i.d. ^b	33	14.5	0.327
Celecoxib 400 mg b.i.d. ^b	30	28.0	0.003

^aCompared to placebo treatment.

^bTwice a day.

Source: Ref. 34.

Table 3 Effect of Celecoxib Treatment on Tumor Multiplicity in the APC Min Mouse Model

Drug	Dose in diet (ppm)	Tumors/mouse	
		Early treatment (30–80 days)	Late treatment (55–80 days)
Vehicle	0	22.4 ± 9.0	22.9 ± 6.8
Celecoxib	150	15.8 ± 9.5	18.0 ± 7.6
	500	15.8 ± 4.6	16.3 ± 6.2
	1500	6.5 ± 4.2	11.1 ± 6.8
Piroxicam	50	5.2 ± 4.0	7.9 ± 4.8

Source: Refs. 34,47,48.

genetic model of human FAP, celecoxib, which selectively inhibits COX-2, showed effectiveness for prevention (i.e., “early” treatment before development of adenomatous polyps) and regression (i.e., treatment after most adenomatous polyps are established), comparable to that of the positive control of piroxicam. Celecoxib caused dramatic reductions in the multiplicity of tumors in a dose-dependent manner (Table 3) (34,47,48).

Additionally, in the rat colon cancer model induced by azoxymethane, treatment with celecoxib for 11 weeks resulted in a 40% reduction in aberrant crypt foci that was similar to that observed for the positive control, sulindac, given at its MTD (320 ppm) (34,49). When administered for one year in the diet, 1500 ppm of celecoxib reduced tumor incidence by 93%, surpassing the results observed in similar studies with various non-steroidal anti-inflammatory drugs (NSAIDs) (34,50).

In the review document, the medical reviewer clearly indicated that the approval of celecoxib for the reduction of the number of adenomatous colorectal polyps in FAP patients was supported by (1) evidence from animal colon tumor models, demonstrating a reduction in the incidence and multiplicity of tumors with its exposure, and (2) numerous clinical studies, mostly small, uncontrolled series, demonstrating the ability of other NSAIDs, notably sulindac, to reduce colorectal polyps in FAP patients (34). This example adequately illustrates how preclinical animal models, when coupled with the well-understood pathophysiology of a disease, can be successfully used to support the efficacy claim for regulatory approval.

3. UTILIZING THE PRECLINICAL DATABASE TO ADDRESS SAFETY CONCERNS

When the FDA reviews a submitted NDA package, any safety concerns raised during the preclinical or non-clinical phase are given special attention

if they were not adequately addressed in the clinical development, or if those clinical studies addressing the same safety issues were limited in terms of scope and exposure time. This can become more serious if the same or similar safety issues were seen in a drug of the same class. For example, cardiac hypertrophy was observed in all animal species tested with pioglitazone (51,52), a thiazolidinedione developed for the treatment of type II diabetes mellitus; this was also seen in preclinical studies with troglitazone (53) and rosiglitazone (54). Since, diabetic patients are obese in general and are likely to have other chronic diseases, such as hypertension and congestive heart failure, cardiac hypertrophy was viewed as one of the main safety concerns by the FDA medical reviewer (55).

In order to directly address this issue, the sponsor conducted a 26-week, placebo-controlled, randomized, double-blind study to compare changes in the echocardiogram in patients with type II diabetes treated with four doses of pioglitazone (7.5, 15, 30, and 45 mg). Patients who had valvular abnormalities, ischemic heart disease leading to left ventricular motion abnormalities, or symptomatic heart failure were excluded, and 80 patients were assigned to each dose group. No significant changes from the baseline echocardiogram were noted, and there were no clinically meaningful differences among the treatment groups. However, the design of this study was criticized by the medical reviewer at the FDA because poor glycemic control in the placebo-treated patients could produce harmful effects on the heart. Additionally, the analysis was not appropriately adjusted for body weight and lipid profiles which could also impact the heart, and more serious patients, i.e., New York Heart Association (NYHA) functional class III or IV, were not included in the study.

Based on this reasoning, the medical reviewer concluded that the cardiac safety concern needed to be clarified further because, as the drug reached the market, patients with different, possibly more serious, profiles from those studied in the pivotal clinical trials would be exposed and the exposure time increased. Likewise, the pharmacology reviewer, concurring with this view, commented that clinical safety should have been demonstrated for approval. Pioglitazone was finally approved by the FDA in 1999; however, the sponsor was requested to conduct a phase IV, randomized, placebo-controlled 6-month clinical study in patients with type II diabetes and NYHA Class II and early Class III congestive heart failure, along with six additional phase IV commitments (56).

The pioglitazone NDA case clearly illustrates how seriously the FDA considers any major safety concerns seen in the preclinical studies in the final NDA review process. Therefore, drug developers should take a serious look at any major safety issues raised during the preclinical phase, and clinical studies should be designed to produce clinically meaningful information to address these issues.

4. UTILIZING THE PRECLINICAL DATABASE TO DESIGN IN VIVO METABOLIC DRUG-DRUG INTERACTION STUDIES

Hepatic metabolism and renal excretion are the two main elimination routes of a non-protein drug or its metabolites (57). Many metabolic pathways, including the hepatic cytochrome P450 enzymes (CYP), can be inhibited, activated, or induced by the administration of a concomitant drug. More and more, preclinical *in vitro* studies using suitable probes and appropriately validated experimental methods, [e.g., human liver microsomes (58,59) or recombinant cytochrome P450 (59,60)] can be used as screening mechanisms to rule out a possible metabolic pathway and drug-drug interactions that could occur via this pathway. In many cases, negative findings from early *in vitro* studies, if coupled with similar results from early clinical studies, can eliminate the need for later *in vivo* clinical investigations (61,62).

On the other hand, when positive findings are seen in *in vitro* metabolic studies, *in vivo* clinical studies are recommended. The clinical importance of the potential drug-drug interaction should be quantitatively estimated for safe and efficient use in patient populations that were not necessarily tested in clinical development, for which the interaction may be sufficiently large to necessitate dosage adjustment or therapeutic monitoring of the drug itself or concomitant medications (61). For example, a potent CYP3A4 inhibitor, ketoconazole, inhibited 85% of *in vitro* pioglitazone metabolism at equimolar concentrations, suggesting that pioglitazone may be a substrate for the CYP3A4 metabolic pathway (63). However, the sponsor did not conduct any *in vivo* drug-drug interaction studies based on these preclinical results; instead, they conducted *in vivo* drug-drug interaction studies of pioglitazone with digoxin, glipizide, warfarin, and metformin, in which no significant changes in PK parameters were found. The sponsor's approach to address possible drug-drug interactions focused mainly on a specific group of drugs that were likely to be coadministered with pioglitazone (digoxin, hydrochlorothiazide) or for which the clinical consequences of potential interactions were of concern.

Additionally, troglitazone, another thiazolidinedione, was known to potentially induce CYP3A4 (64), but pioglitazone was not tested for this potential, neither *in vitro* nor *in vivo*. The clinical pharmacology and biopharmaceutics reviewer at the FDA discussed that the drug interaction potentials of pioglitazone were not investigated using the understanding of the metabolic pathways of related agents; instead, the sponsor's approach made it hard to draw conclusions for general drug-drug interaction profiles (62,63). As a result, the FDA requested that the sponsor conduct two drug-drug interaction studies as the phase IV commitments for regulatory approval of pioglitazone: a two-way crossover drug interaction study of single-dose pioglitazone and single-dose ketoconazole (i.e., pioglitazone as a

substrate of the CYP3A4 pathway), and a two-way crossover drug interaction study of steady-state pioglitazone and single-dose midazolam (i.e., pioglitazone as a possible *inducer* of the CYP3A4 pathway) (56).

Again, this pioglitazone drug interaction study exemplifies why it is so important for clinical drug development to be guided by information and knowledge obtained from preclinical studies.

5. LIMITATIONS AND PREDICTIVE VALUE OF THE PRECLINICAL DATABASE

As presented in the previous sections, the preclinical database can play a significant role in clinical drug development by providing supportive, sometimes critical, information on the safety and/or efficacy of the drug in humans for the purposes of regulatory approval. Although new *in vitro* techniques have reduced the need for animal studies, these conventional studies still have been the primary method to understand the *in vivo* pharmacology, toxicology, efficacy, and safety of a drug for clinical development.

However, toxicity testing using animals carries ethical liability, is time consuming and expensive [for example, a monkey can cost \$3500–\$5000 (65)], which together may restrict the breadth of the preclinical database for clinical drug development (66). Additionally, there are some inherent limitations of preclinical studies, which may render a naïve empirical extrapolation from animals to humans less useful or even meaningless for the purposes of an informed decision during clinical drug development (67). For example, the variability between human individuals can be overestimated due to extrapolation across different animal species; diversity even exists within inbred strains of homogenously derived and maintained laboratory animals (68,69). Furthermore, there are few animal models or animal-based *in vitro* or *ex vivo* systems that can duplicate the structure or function of humans, and drug developers must validate the animal systems as models for human systems at all levels (70–72).

Therefore, the value of animal models in predicting the efficacy and toxicity of a drug in humans can be realized only in cases where findings are congruent in both animal models and humans (73,74), i.e., efficacy/toxicity found (75–77), or not found (78), in both animals and humans (Table 4). When animal models failed to define a drug's efficacy and toxicity, seen later in humans (third row of Table 4), these animal models had no predictive value (79); in practice, the drug could have dropped from further clinical development at preclinical stage. But, the failure to find efficacy in animal models does not necessarily preclude further clinical development. For example, none of animal models of osteoarthritis (OA), including mouse and guinea pig spontaneous OA, meniscectomy and ligament transection in guinea pigs, meniscectomy in rabbits, and meniscectomy and cruciate transection in dogs, have been used with success to predict drug efficacy

Table 4 Predictive Value of Animal Models

Findings in		Predictive value	
Animal ^a	Human ^b	Efficacy	Toxicity
Found	Found	Yes	Yes
Not found	Not found	Yes	Yes
Not found	Found	No	No
Found	Not found	No	?

^aMultiple of human dose.

^bHuman effective dose.

Source: Ref. 73.

in humans, because no agents have been shown to provide anything other than symptomatic relief in human OA (80).

Of course, if the efficacy of a drug was seen in animal models, but not found in humans, the drug would fail to show proof of concept in humans, and is likely to be dropped from further development. The most difficult decision, however, has to be made when toxicity, found in an animal model, is not necessarily seen in humans, after which the predictive value of this animal model may be questioned. In this case, drug developers must carefully plan and design clinical studies to adequately address safety issues raised in animal models, and the preclinical database can give them meaningful insight into potential safety concerns, as exemplified in the case of pioglitazone (51,55,56).

Physiologically based pharmacokinetic (PBPK) modeling, although not widely used in drug development because of its technical complexity, may be useful for internal decision making to assess the predictive value of the preclinical database for clinical drug development (81–83). Because PBPK approaches help drug developers link toxicity data from animal species to expected clinical observations using the exposure–response (i.e. toxicokinetic–safety) relationship, PBPK modeling can serve as a valuable tool for understanding what preclinical PK, safety, and efficacy results ultimately mean in humans (84).

6. CONCLUSIONS

Although there are some limitations of preclinical studies, they provide the sole source of data upon which the assessments of drug efficacy and safety are to be made before human data become available (73). Therefore, if utilized adequately, coupled with more physiological models that account for the biological inter- and intra-species diversity, and variability based on

mechanistic understanding of a drug action, the preclinical database is a valuable tool to support an efficient and informative clinical drug development which leads to a successful regulatory approval.

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